FACTORS AFFECTING THE CHEMICAL COMPOSITION OF MILK

A Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science

by

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An abstract of a thesis submitted to the
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by Radhaballabh Chanda, a candidate
for the degree of Ph. D.

A detailed study has been made of the effect of thyroxine and thiouracil on the composition of the milk of both cows and goats. Certain variations which occur in the composition of human, cows' and goats' milk as lactation progresses have also been studied. The thesis opens with a general introduction explaining the object of the work, and the results are recorded in four chapters. Each chapter contains an introduction, a section on the experimental work, a discussion and summary. The thesis ends with a list of references.

Chapter I. The effect of thyroxine and thiouracil on the partition of phosphorus and aneurin in cows' milk and the relation of various phosphorylated compounds with phosphatase.

The effects of thyroxine on the partition of phosphorus were investigated and it was found that the hormone produced very large increases in the ester-P and lipid-P in cows' milk. Inorganic-P was at the same time decreased. A novel feature of the present experiments was the use of thiouracil to produce hypothyroid lactating animals. The partition of phosphorus in the milk of the hypothyroid cows showed changes which were the reverse of those produced by thyroxine, and it is important to note that, though it is only a minute fraction of the ester-P present in milk, aneurin-pyrophosphate (cocarboxylase), was found to be increased by thyroxine and decreased by thiouracil. All these changes were closely correlated with the phosphatase which was increased by thiouracil and decreased by thyroxine. The relationship between the enzyme and phosphoric esters found in the milk of cows treated with thyroxine or thiouracil held in normal cows throughout lactation. Neither thyroxine nor thiouracil affected the total nitrogen in the milk, but the phosphorus content was increased by thyroxine and decreased by thiouracil so that the ratio of N/P was affected by the hormone. It was found that normally the N/P ratio in milk was smallest when the cow was approaching the peak of lactation and that it increased in late lactation.

Chapter II. The effect of thyroxine and thiouracil on the concentration of the major constituents and on some of the water-soluble vitamins in cows' milk

Thiouracil decreased the milk yield of lactating cows. Its effect was thus the reverse of that of thyroxine. Thyroxine caused an increase in the fat and solids-not-fat contents of milk, but its effect on fat was more marked and less variable than its effect on solids-not-fat; but neither of these two effects was constantly reproducible. There was a small increase in the lactose content of milk in the thyroxine treated cows corresponding to a simultaneous decrease in the chloride content. Thiouracil caused a reverse effect in these constituents so that there was a constant relationship between lactose and chloride. The calcium content of the milk was unaffected by either drug. The ascorbic acid content was increased by thiouracil and decreased by thyroxine but the riboflavin content remained unchanged.

Chapter III. The metabolism of carotenoids and vitamin A in lactating cows and goats with special reference to nutritional and hormonal effects on the secretion of vitamin A in their milk

This chapter is divided into two sections. In Section 1 it is shown that the digestibility of carotene in goats determined with the aid of Cr_2O_3 as a marker was in close agreement with that obtained by the direct method. By applying the marker method for determining digestibility it was further shown that the goats digested carotene more efficiently than the cows. In Section 2, the effects of thyroxine and thiouracil on the digestion of carotene are described. Thyroxine increased and thiouracil decreased the digestibility of carotene in both cows and goats, but the increases caused by thyroxine were larger in the cows than in the goats. On the other hand thiouracil reduced the digestibility more in goats than in cows.

The effect of thyroxine on the vitamin A and carotene content of the milk are also described in this section. Thyroxine increased the vitamin A content of the milk fat of both cows and goats, but neither drug caused any carotene to appear in the goats' milk. The carotene content of cows' milk was, however, increased by thyroxine and decreased by thiouracil. Cows deprived of carotene showed an increase in the alcoholic form of vitamin A in their milk. Thyroxine which is known to accelerate hepatic depletion caused a dramatic increase in the vitamin A alcohol in the milk of cows deprived of carotene. Carotene is not normally present in goats' milk but the presence of β-carotene was demonstrated in the colostrum and the liver of the goats.

Chapter IV. The chemical composition of human milk with particular reference to the contents of fat, solids-not-fat, protein, carotenoids and vitamin A and the relation of phosphatase to the partition of phosphorus and aneurin

The phosphatase content of human milk was found to be very low compared with that of cows' milk, and the proportions of the total phosphorus present in the form of lipid and ester were larger in human milk. The cocarboxylase content was also greater and the free aneurin smaller so that the relationships between the enzyme and various phosphoric esters in human milk were similar to those found in cows' milk. It was shown further that in respect to these constituents, goats' milk resembled human milk more than cows' milk. The secretion of α-carotene, lycopene and lutein along with β-carotene was demonstrated in human milk. The proportion of vitamin A present in the alcoholic form was greater in human milk than in cows' milk.

The relation of new observations reported in this thesis to current biochemical theories of milk secretion are discussed in the appropriate chapters of the thesis.

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General Introduction

Early work on the effect of thyroxine on milk yield and composition was done by Graham (1934 a, b) and Folley & White (1936). The effect of thyroxine on the composition of milk and on the nature of milk fat was studied at the Hannah Dairy Research Institute about 12 years ago by Smith & Dastur (1940). A few years later similar investigations were continued by Owen, and his results on the effect of thyroxine on the composition of milk and on the metabolism of nitrogen, calcium and phosphorus were published in 1948 (Owen, 1948 a,b). The work in the present thesis is a continuation of these earlier investigations. Thyroxine injections have been used in all the experiments in spite of the widely favoured and much publicized use of iodinated casein. The advantage of using thyroxine itself was that an accurate dosage of the hormone could readily be given and that there was no risk of giving excessive amounts of iodine or other substances as there is with iodinated proteins. Bartlett, Rowland & Thompson (1949) reported that a cow ingesting 20 g. iodinated casein per day consumes as much as 1.4 g. iodine in the 24 hr., whereas in the present experiments only 10 mg. of thyroxine itself were given per day and only 65% of that would be iodine.

During the past 50 years chemical analyses of milk of many species have become available, and from time to time various workers have collected the results together to see if they could assign reasons for

changes of composition from species to species. Thus Bunge (1902) drew up a table of milk composition and concluded from it that the protein content of the milk was correlated with the time taken by the young to double their birth weight. The mineral composition seemed to bear out the same generalization. Bleyer (1930) and Davies (1936) brought Bunge's table more up to date and made similar conclusions. An additional conclusion, later pointed out by Kay (1937), was that the fat concentration of the milk may be correlated with the coldness of the environment into which the young are born. A more complete table of the composition of various species of animals was later drawn up by Brody (1945).

In 1939 (Ludwig & von Mutzenbecher, 1939) it was discovered in Germany that proteins treated with iodine under certain well defined conditions could acquire thyroid activity. This activity was shown by Harington & Pitt-Rivers (1939) to be due to the production of l-thyroxine in the proteins. Turner and his colleagues (Turner & Reineke, 1944) in America further perfected the production of iodinated casein by a patented process which guaranteed its thyroid activity. This material was extensively used in both America and Britain for experiments designed to increase the yield of milk per cow. Thyreo-protein (including synthetic thyroxine, which is now cheaper than iodinated casein) gives large and fairly well sustained increases in the yield of milk, and these are usually accompanied by

increases in the percentage of fat. The use of thyroid-active material, except under expert supervision, has not been permitted because long-term effects on the cow and on the foetuses which they may carry when they are being treated, are still under investigation. Nevertheless much biochemical work has been directed to the investigation of changes produced by thyroxine in the composition of milk. It was found, however, that except for changes of the percentage of fat, minor changes in the percentage of lactose, and except for a change in the ratio of total to free aneurin, thyroxine was without effect on the composition of milk (Blaxter, Reineke, Crampton & Petersen, 1949). Partition of protein was unaltered (Van Landingham, Hyatt & Weakley, 1946). It was, therefore, generally believed that thyroxine had no major effect on milk composition. Results reported in the present thesis show that this generalization does not hold for some of the components which are present in milk in relatively small amounts but which are of great importance nutritionally. The present experiments demonstrate that thyroxine has a far-reaching effect on the partition of phosphorus in the milk, although in the same experiments no changes of nitrogen partition or of total calcium, could be demonstrated. They further show that the metabolism of carotene from its absorption by the goat, or the cow, to its appearance as vitamin A in the milk, is extensively affected by thyroxine. An attempt has also been made to correlate these changes induced by treatment

with thyroxine, with biological changes naturally occurring in milk at the inception of lactation and with changes occurring towards the end of lactation. Differences in the composition of milk of different species of animals have also been investigated. To throw the quite marked changes caused by thyroxine into greater relief, the anti-thyroid drug, thiouracil, which has been extensively used for treating toxic goitre in humans, has been used in all the present experiments, and it has been found that for any effect of thyroxine on the composition of milk, the opposite effect can be generated by thiouracil.

As a result of the experiments reported in this thesis, certain generalizations about milk secretion have become possible and these are dealt with in the discussion at the end of each chapter.

CHAPTER I

The effect of thyroxine and thiouracil on the partition of phosphorus and aneurin in cows' milk and the relation of various phosphorylated compounds with phosphatase

Phosphatases are those enzymes which catalyse the scission or formation of ester linkages. The natural substrates for their activity are such esters as glycerophosphates, hexose phosphates and nucleotides. The presence of alkaline phosphatase in milk was established by Graham & Kay (1934). Folley & Kay (1935) also found that the secreting cells of the mammary gland are strikingly rich in the enzyme. The marked changes in the concentration of alkaline phosphatase in the rat mammary gland during pregnancy, lactation and involution has been studied by Folley & Greenbaum (1947). It is, however, not known what function phosphatase plays in the lactating mammary gland. Folley (1949) indicated the possibility of phosphatase being concerned in the synthesis of milk protein. It has also been pointed out by Moog (1946) that phosphatases are often found in cells in which active protein synthesis is proceeding and that in such cells there is frequently a correlation between the content of nucleic acid, thought to be an instrument of protein synthesis, and phosphatase.

Folley & Kay (1936) have shown that phosphatase in cow's milk increases gradually throughout lactation after having passed through a minimum value in the first 2 or 3 weeks of lactation. They stated that the amount

of phosphatase secreted in unit volume of milk may be regarded as the inverse index of the efficiency of the mammary gland so that the phosphatase curve throughout lactation is the inverse of that of the lactation curve. Kannan & Basu (1948) have extended the observation of Folley & Kay (1936) in cattle to include sheep, goats and buffaloes in India. In all these species the phosphatase concentration in the milk increases as lactation advances. Weil (1941) made the interesting observations that, following parturition, the plasma phosphatase in rats was markedly increased. So far no specific function of phosphatase in milk itself has been discovered. It is known, however, that phosphorus in milk is present in the form of inorganic phosphate and in organic compounds such as esters, casein and lipids (Graham & Kay, 1934).

Folley & White (1936) found an increase in the phosphatase content of serum from cows treated with thyroxine, and this was accompanied by a very large decrease in the milk phosphatase. Thyroidectomy decreased the serum phosphatase in rats (de Luca, 1940). Owen (1948 a, b) showed that the percentage of phosphorus in the milk of cows treated with thyroxine was increased without affecting the nitrogen or calcium contents. It was also observed by him in detailed metabolism trials that thyroxine decreased the retention of nitrogen in lactating cows. It also increased the loss of calcium to such an extent that more calcium was excreted in the faeces than was present in the food so that some of the

calcium in the faeces must have been supplied by the skeletal stores. Under these conditions the parodoxical observation was made that thyroxine caused the lactating cow to retain more phosphorus than under normal circumstances, with the result that the metabolism of phosphorus followed neither the metabolism of the nitrogen nor that of the calcium.

Experiments were therefore planned with the object of partitioning the phosphorus in the milk of cows treated with thyroxine to find whether any particular types of phosphorus compounds were responsible for the increase of total phosphorus in the milk and whether any biological significance could be attached to their change in conjunction with the simultaneous change in the enzyme phosphatase. To throw into greater relief any chemical differences between the milk of control cows and those receiving treatment with thyroxine, a third group of cows was treated with the antithyroid drug, thiouracil.

Observations made later on the partition of aneurin in relation to phosphatase are also reported in this chapter. Investigations on the relationship of the enzyme with various phosphorylated compounds in colostrum, and in milk from cows at various stages of lactation, were also carried out. The experiments will be described in the order in which they were made.

Experimental

(A) Phosphatase and partition of phosphorus

Two experiments were made to study the effect of thyroxine and thiouracil on the partition of phosphorus in relation to phosphatase. For each experiment cows with comparable milk yields and in mid-lactation (14 - 16 weeks post partum) were chosen. The cows were all of Ayrshires from the Kirkhill herd. Three cows were used for the first experiment and five for the second one. Each experiment lasted 9 weeks and was divided into three equal periods. In each experiment the design was the same in that a period of 3 weeks during which hormonal treatment was given (Period 2) was preceded by a preliminary control period (Period 1) and was succeeded by recovery period (Period 3). Some cows were kept as untreated controls, i.e. they did not receive any treatment in period 2. The composition of the milk during treatment could therefore be compared with the composition of the milk of the same cow before and after treatment and with the milk which the untreated cow was secreting simultaneously. All the cows were identically managed.

A readily reproducible effect of treating lactating cows with thyroxine is a sudden and sustained decrease in the concentration of phosphatase in the milk (Folley & White, 1936; Owen, 1948 a), while phosphatase concentration in milk is known to be increased with advancing lactation (Folley & Kay, 1936). In the present experiments therefore cows were chosen with dates of

calving as near as possible to one another so as to give approximately equal initial concentrations of phosphatase in their milk. The initial concentration of phosphatase in the milk can be seen in Fig. 1. In experiment 1 one of the cows received 10 mg. thyroxine per day and another one 10 mg. thiouracil per day. The third cow was kept as an untreated control. In experiment 2, one cow acted as untreated control. The remaining four cows were divided into two pairs. One pair received 10 mg. thyroxine daily and the second pair received 20 mg. thiouracil daily during period 2. When these experiments were envisaged the author had no knowledge of the effects of injecting the goitrogenic drug thiouracil into lactating animals of any species. It was known, however, that 10 mg. thyroxine daily approached the maximum safe dose for a lactating Ayrshire cow (Owen, 1948 a). By comparison with this injected amount, the amounts of thiouracil given by mouth to human patients suffering from toxic goitre, 100 - 500 mg. (Brit.Pharm.Codex, 1949) appeared to be very large. It was therefore decided to use the phosphatase titre of the milk to compare the relative potencies of thiouracil and thyroxine. In both experiments, the phosphatase in milk was determined routinely. In experiment 1, the effect of 10 mg. thiouracil daily was compared with the effect of 10 mg. thyroxine. On the basis of the response in phosphatase in experiment 1, the dose of thiouracil was doubled in experiment 2.

Milking was done by machine in the milking parlour attached to the dairy, and 2 lb. of a well

mixed milk sample from each cow was taken every second day for analysis. On these samples fat, total solids, lactose, chloride, freezing point, partition of phosphorus and partition of nitrogen were determined. In this chapter the results for phosphatase, total N, total P, partition of N, and partition of P are reported. The proximate content in the milk of some water-soluble vitamins will be dealt with in the next chapter.

(B) Phosphatase in relation to partition of aneurin

The partition of aneurin in relation to phosphatase was studied in a third experiment (Experiment 3) in which six cows were used. They were, however, in earlier stages of lactation (6 - 8 weeks post partum) than those used in experiments 1 and 2. The plan of the experiment was the same. A hormone treatment period of 3 weeks (Period 2) was preceded by a preliminary control period of 3 weeks (Period 1) and was succeeded by a final recovery period of 3 weeks (Period 3). The cows were divided into three pairs. One pair received 10 mg. thyroxine daily and a second pair received 20 mg. thiouracil daily during period 2. The third pair did not receive any treatment in any period and thus acted as untreated controls.

Milk samples were collected every second day, phosphatase was determined, and aneurin was partitioned into various fractions.

Administration of the drugs

A solution of 'thyroxine sodium B.P.' (British Drug Houses, Ltd.) containing 1 mg./ml. was made up according to Folley & White (1936). The rather insoluble material was dissolved in a minimum quantity of $N/_{10}$NaOH and neutralised to the point of reprecipitation with $N/_{10}$HCl. It was made up to volume with distilled water and pasteurised. A solution of thiouracil was made up in a similar manner. It was observed that thiouracil is more readily soluble in freshly prepared caustic soda. Accordingly, a weighed quantity of thiouracil was dissolved in a minimum excess of freshly prepared $N/_{10}$NaOH and neutralised to phenolphthalein with $N/_{10}$HCl. The solution was made up to volume and pasteurised before being injected. The thiouracil solution was prepared so as to contain 1 mg./ml. in experiment 1 and 2 mg./ml. in experiments 2 and 3. Each day during the hormone treatment periods, 10 ml. of these solutions were injected subcutaneously into the flanks of the cows. The injections were given immediately after evening milking. The solutions of the drugs were preserved in cold storage and brought to room temperature before use.

Diet

The animals were kept in the byre during the experiments and were fed on the ration which the remainder of the herd were receiving. The maintenance requirement was provided by hay. The production ration

consisted of a mixture containing 30% bean meal, 40% bruised oats and 30% grass meal. The requirements were calculated on the basis of 0.6 lb./digestible crude protein (D.C.P.) and 6 lb. starch equivalent (S.E.) for maintenance of 1000 lb. body weight, and 2.8 lb. S.E. and 0.6 lb. D.C.P. per gallon of milk production.

Methods of analysis

Total N.

This was determined by the macro-Kjeldahl method using 5 ml. milk. The milk was carefully mixed before weighing, and was digested with 20 ml. concentrated sulphuric acid and 1 g. of a mixed catalyst consisting of K_2SO_4 (40 parts), $CuSO_4$, $5H_2O$ (5 parts) and powder selenium (1 part) ground together in a mortar. The ammonia liberated on making the digest alkaline was trapped in the usual way in a known volume of $N/_{10}$ H_2SO_4 and determined by back titration.

Phosphatase

Phosphatase was determined by Neave's modification (1939) of the Kay & Graham (1935) test. The short test was used. The precautions discussed in the technical communication of the Imperial Bureau of Dairy Science (Kay, Aschaffenburg & Neave, 1939) were strictly followed. The buffer substrate solution was made freshly from tablets (British Drug Houses, Ltd.) before each test. Two tablets were dissolved in 100 ml. distilled water and the resulting solution was saturated with

pure chloroform. Folin-Ciocalteu reagent (British Drug Houses,Ltd.) was diluted with twice its volume of 5% (w/v) solution of sodium hexa-metaphosphate (Neave, 1939).

To 10 ml. of the buffer substrate solution contained in a 25 ml. test tube, 0.5 ml. of the well mixed milk sample was added. It was mixed thoroughly and incubated at 47° for 10 min. in a water-bath. The tube was removed and cooled by immersion in cold water at 15-20°. When the mixture was cold, 4.5 ml. of diluted Folin-Ciocalteu reagent was added, the tube was shaken and then allowed to stand for 3 min. The contents were then filtered, 10 ml. of the filtrate pipetted immediately into a test-tube and 2 ml. 14% (w/v) pure anhydrous sodium carbonate (standardised by titration) added. The tube was placed in a boiling water-bath for 2 min. and cooled by immersion in cold water. The blue colour developed was read against a control in the Spekker photoelectric colorimeter using a red filter.

For the control test, 10 ml. of buffer substrate solution were taken in a test-tube, and 4.5 ml. of Folin-Ciocalteu reagent and 0.5 ml. milk were added. The mixture was allowed to stand for 3 min. and filtered. The colour was developed as before using 10 ml. of the filtrate.

The amount of phenol liberated was calculated using a calibration curve prepared in the following way. The phenol solution was standardised by treating a 0.1%

solution of pure phenol with an equal volume of 0.1 N iodine in an alkaline medium. The excess of iodine was determined by back titration with sodium thiosulphate after adding excess of conc. HCl. (See Hawk, Oser & Summerson, 1947, p.879). On the basis of the standardisation a working standard solution was prepared so as to contain 0.02 mg. phenol/ml. A calibration curve was drawn in the range of 0.01 - 0.2 mg. phenol.

The results for phosphatase have been recorded as mg. phenol liberated by the phosphatase in 100 ml. milk. Such units are reproducible but empirical and have therefore been called 'arbitrary units' in the tables and figures.

Partition of Phosphorus

Phosphorus was partitioned by a slight modification of the method of Graham & Kay (1934). Final estimations of the total P and the phosphorus of each fraction were by the method of Fiske & Subbarow (1925) using a red filter in the spekker photoelectric absorptiometer. Working details for preparing the standard phosphate solution and various reagents are described by Hawk, Oser & Summerson (1947). Essential details for the partition of phosphorus are given below.

Total P.

Phosphorus was brought into aqueous solution by the method of Horecker, Ma & Haas (1940). The milk (10 ml.) was weighed in a 100 ml. volumetric flask and diluted to the mark with distilled water. After the

contents had been thoroughly mixed, 3 ml. were pipetted into a micro-Kjeldahl flask, 1.5 ml. concentrated H_2SO_4 added and the mixture heated till it became brown. To the cooled mixture a few drops 30% H_2O_2 (B.D.H., M.A.R.) were added and heating was continued. This last process was repeated when necessary till the mixture was colourless. The cooled solution was transferred to 100 ml. flask with distilled water, and the blue colour developed as described by Hawk, Oser & Summerson (1947).

Total acid-soluble P.

Into a 50 ml. volumetric flask 5 ml. milk were weighed. After addition of 25 ml. distilled water the contents were mixed and made to the mark with 25% (w/v) trichloroacetic acid previously cooled in the ice-box at 4-5°. The flask was then shaken and left to stand for 5 min. After filtration through a Whatman No.42 ashless filter paper, 3 ml. of the solution were pipetted into a micro-Kjeldahl flask and the P determined as before.

Inorganic phosphate

Immediately after filtration 15 ml. of the trichloroacetic acid extract (used for determining total acid-soluble phosphorus) were pipetted into a 50 ml. centrifuge tube and neutralised to phenolphthalein (pH 8-9) with saturated baryta. After addition of 2 ml. 20% solution of barium acetate the mixture was left in the ice-box for 20 min. It was then centrifuged to pack the precipitate firmly. The supernatant fluid

was discarded and the precipitate was washed with 10 ml. of a 1% solution of barium acetate. The wash liquid was discarded, the precipitate treated with one drop of concentrated H_2SO_4, and then diluted with about 20 ml. distilled water. The solution and precipitate were quantitatively transferred to a 50 ml. volumetric flask, made to volume and filtered. The P was determined as before. As a technical check inorganic-P in some of the samples was determined directly in 3 ml. of the unhydrolysed trichloroacetic acid extract. Table 1 demonstrates the agreement between the two methods.

Ester-P

This was calculated from the difference between the inorganic P and the total acid-soluble P.

Table 1. The ester-P content of milk determined as:- (a) the difference between the total acid-soluble P and the Ba precipitable P, and (b) the increase of phosphate on acid hydrolysis

Sample No.	Ester P (mg. P/100 g. milk)	
	(a)	(b)
1	7.8	7.4
2	6.2	6.0
3	8.1	7.5
4	9.2	8.7
5	9.5	9.3
6	6.1	6.4
7	8.1	7.9
8	7.3	7.5
9	7.5	7.8
10	8.3	7.9
Mean	7.81	7.64

"t" for paired difference $= \frac{\text{Mean diff.}}{\text{S.E.of diff.}} = \frac{0.17}{0.1044} = 1.628$ (N.S.)

Lipid P.

Into a 100 ml. volumetric flask 2 ml. milk were weighed. To this were added 70 ml. of a solution of 1 volume of di-ethyl ether in 3 volumes of ethanol, and the mixture was heated in a water-bath at 70° for 15 min. The flask was then cooled in water and the contents were made to the mark with the ether-ethanol solution. The extract was filtered into a 25 ml. cylinder, the filtration being done rapidly to avoid loss by evaporation. Filtration was discontinued as soon as 25 ml. filtrate had been collected. The filtrate was evaporated a little at a time in a micro-Kjeldahl flask till all of it had been transferred. The flask was finally washed with a little more of the ether-ethanol solution so as to ensure a quantitative transfer. All these operations were completed quickly to avoid any precipitation of the dissolved phospholipins. After all the solvent had evaporated the residue was heated with 1.5 ml. 10N H_2SO_4 and 30% H_2O_2 (B.D.H., M.A.R.). Several repetitions of the addition of H_2O_2 were necessary before a water-clear solution resulted. The clear solution was transferred quantitatively to a 25 ml. volumetric flask. After addition of 2.5 ml. Molybdate III (Hawk, Oser & Summerson, 1947) and 1 ml. 1:2:4 amino-naphthol-sulphonic acid reagent, the mixture was made to volume and the resulting blue colour read as for other fractions.

Casein P.

This was calculated from the equation

$$\text{Casein P} = \text{Total P} - (\text{Total acid-soluble P} + \text{lipid P})$$

It was possible to check the reliability of the methods for ester-P and casein-P by other independent methods. The casein method was checked by direct precipitation of casein in the presence of an acetate buffer at pH4 from 10 ml. milk as described by Rowland (1938) but in a 100 ml. centrifuge tube. The tubes were spun in the large International Centrifuge for 30 min. at 2000 r.p.m. The liquid was discarded and the precipitate was washed with 50 ml. ether and centrifuged again. The washing with ether was repeated twice (i.e. three times altogether). The ether-washed casein was dissolved in a minimal amount of ammonia and the solution transferred to a 50 ml. measuring flask and made to volume with distilled water. After neutralisation with N H_2SO_4 and treatment with H_2SO_4 and H_2O_2 the P content of 5 ml. of this solution was determined as already described. Table 2 presents evidence of the agreement of results for casein-P in milk obtained by the two different methods.

Table 2. Casein P in milk determined (a) directly in the casein precipitate, and (b) from the difference between the total P and the total non-casein P.

Sample No.	Casein P (mg./100 g. milk)	
	(a)	(b)
1	18.4	17.6
2	17.5	18.1
3	15.9	14.5
4	17.3	18.1
5	16.8	17.5
6	16.2	15.4
7	18.9	19.2
8	17.4	15.9
9	18.3	19.1
10	19.5	18.3
Mean	17.62	17.37

"t" for paired difference = $\frac{0.25}{0.3077}$ = 0.8125 (N.S.)

Partition of nitrogen

To obviate the necessity of laboriously washing any precipitate, the various protein fractions were determined from analysis of nitrogen in filtrates only, pipettes being specially calibrated when delivering solutions saturated with salt. Globulin was determined by the difference between casein precipitated with acetate buffer as described by Rowland (1938), and casein + globulin precipitated as described by Elsdon & Walker (1942). Other protein precipitations were carried out exactly as described by Rowland (1938). The total N in each filtrate was determined by micro-Kjeldahl using the Wagner-Parnas still. When digesting the filtrate with H_2SO_4 a mixed catalyst as mentioned under total N was used except for the filtrate from the albumin + globulin precipitation which was so rich in

$MgSO_4$ that a catalyst containing only $CuSO_4$ and Se was used, the $MgSO_4$ itself serving to raise the boiling point of the H_2SO_4. Ammonia was trapped in 2% boric acid and titrated directly with 0.02N H_2SO_4. The boric acid solution contained both methyl red and bromcresol green.

Creatine and creatinine

Creatinine was determined by treatment of the tungstic acid filtrate of milk with alkaline picrate solution, the resulting colour being read in a Spekker photoelectric absorptiometer using a green filter. Creatine was determined by subtracting the preformed creatinine value from the total creatinine determined by the same method after autoclaving the protein-free filtrate with HCl. The procedures followed were as outlined by Hawk, Oser & Summerson (1947).

Partition of aneurin

Aneurin in milk was partitioned by a slight modification of the method described by Houston, Kon & Thompson (1940). The final estimations were made fluorimetrically by the method of Jansen (1936).

Free aneurin

Three 10 ml. samples of skimmed milk were pipetted into 50 ml. glass stoppered cylinders. Methanol (1 ml.) was added to each, and to the first and second cylinders 0.4 and 0.5 ml. of freshly prepared 1% $K_3Fe(CN)6$ was also added, the third cylinder

being kept as a blank. To each was added 5 ml. of 40% NaOH, followed after 1 min. by 25 ml. of isobutanol. The mixtures were then well shaken and allowed to stand. When the layers were clearly separated, the lower layer was drawn off by suction, and 1 g. anhydrous sodium sulphate added. After a short time the clear isobutanol extract was decanted and the fluorescence measured against a quinine sulphate solution (0.05 p.p.m.). The calibration curve was prepared in a similar manner using crystalline aneurin standard.

Trichloroacetic acid soluble aneurin

100 ml. of skimmed milk were pipetted into a 250 ml. pyrex centrifuge bottle and 70 ml. 2% trichloroacetic acid added. The mixture was heated in a boiling water bath for 30 min., cooled and centrifuged at 2000 r.p.m. for 50 min. The clear liquid was poured into a 200 ml. volumetric flask. The protein fraction was stirred with 50 ml. cold 2% trichloroacetic acid and centrifuged again. The extract was combined with the first extract and adjusted to pH4. After making up to volume (200 ml.) 50 ml. of the extract were pipetted in duplicate into conical flasks and 60 mg. takadiastase (Parke, Davis & Co.) added to each. After adding a few drops of chloroform, the mixture was incubated at 37° overnight.

Jansen's method was applied after incubation for the determination of aneurin. The difference between the trichloroacetic acid-soluble aneurin and free aneurin was taken as the cocarboxylase fraction.

Protein-bound aneurin

The protein precipitate left in the centrifuge tube after trichloroacetic acid extraction was stirred with 100 ml. ethyl ether and centrifuged. The ether was poured off and the precipitate suspended in 40 ml. $\frac{N}{100}$ HCl. 0.5 g. pepsin was added, the pH adjusted to 2 and the mixture incubated at 45° for 2 days. The pH was readjusted after the first 24 hr. and 0.5 g. fresh pepsin was added. After incubation, the mixture was centrifuged and Jansen's method was applied as before.

The presence of any aneurin in the enzymes was checked by incubating with takadiastase at pH4 and with pepsin at pH2, and then applying Jansen's method. No measurable quantity was found.

Total aneurin

To 100 ml. of skimmed milk in a 250 ml. centrifuge bottle, 250 mg. of takadiastase (Parke, Davis & Co.) were added and the mixture shaken thoroughly. The mixture was then adjusted to pH4 by adding 5 ml. of N-HCl. The pH was checked and the mixture incubated overnight at 37°. after addition of a few drops of chloroform. Jansen's method was applied to the incubated samples for the determination of total aneurin. Incubation of the mixture for a further 48 hr. with pepsin did not increase the total aneurin value to any significant extent. It was therefore decided to omit this step for all routine estimations.

Results

Estimation of equivalence of the responses of phosphatase to thiouracil and thyroxine

Since the way in which thiouracil inhibits thyroid activity is a matter for speculation (Young, 1944; Andik, Balogh & Donhoffer, 1949) it was empirically assumed at first that weight for weight thiouracil would have an equal but opposite effect to thyroxine. In experiment 1 therefore the effect of equal amounts of these two drugs on the phosphatase content of the milk of three cows was studied. One of these cows, Joyce, received by subcutaneous injection 10 mg. thyroxine per day, the second cow, Sunshine, received 10 mg. thiouracil daily, while the third cow, Gertrude, acted as untreated control. The effects of these treatments on the phosphatase in milk is shown in Fig. 1 which demonstrates that the initial phosphatase titres became widely divergent as a result of the treatment. The phosphatase of Joyce rapidly diminished by comparison with slight rise shown by the untreated cow. The phosphatase of Sunshine, as was expected, increased but at only about half the rate at which that of Joyce decreased. On cessation of the treatments these effects were both reversed so that all three cows secreted milk once more with normal phosphatase titres. From the fact that the rate of increase of the phosphatase titre of the milk of the cow which received 10 mg. thiouracil was about half of the rate of decrease shown by the thyroxine animal, the thiouracil dose in

experiment 2 was doubled. Fig.2, shows that the effect of this doubling was to produce rates of increase of phosphatase in the two thiouracil cows which were practically equal to the rates of decrease of phosphatase in the two thyroxine cows. Furthermore, as in experiment 1, in all five cows the phosphatase titres eventually became the same as one another when the effects of the drugs had worn off. On the basis of these experiments it was assumed that 20 mg. thiouracil were equivalent in the magnitude of its effect to 10 mg. thyroxine. These doses were used in all subsequent experiments.

The results obtained for the effect of the drug on the phosphatase titres during the treatment period (Period 2) have been analysed in Table 3. The data in the third column represent the slopes of the straight lines fitted to points shown in Figs. 1 and 2 by the method of least squares. The fourth column represents the intercept of the line of the third column on the phosphatase axis, i.e. it is a corrected initial titre of phosphatase. The figure for the control cows in the third column of each experiment shows the change of phosphatase which occurred due to progressing lactation. In experiment 1, judging either by total response or by the maximal response 10 mg. thiouracil were about half as potent in changing the phosphatase titre in the milk as 10 mg. thyroxine. From the results of experiment 2 in which the dose of thiouracil was double, it can be seen that judged by

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the same criteria, 20 mg. thiouracil gives an equal but opposite effect to that of 10 mg. thyroxine (Table 3). The size of the maximum response and the number of days required to achieve it are recorded in the last two columns of Table 3. It will be seen that the maximum value in the cow receiving 10 mg. thiouracil was only 198 units compared with 254 and 271 units in the two cows receiving 20 mg. thiouracil, but in the cow receiving the lower dose the maximum value was attained seven days earlier than with the other two cows.

Table 3. The rate of change in the concentration of phosphatase in the milk of cows receiving thyroxine or thiouracil

(arbitrary units)

Cow	Treatment	Response per day during the injection period	Intercept of regression line (theoretical initial titre)	Phosphatase on the day of maximal response	No. of days required to reach maximal response
		Experiment 1			
rtrude	None	+ 0.37	109.3	-	-
yce	10 mg. thyroxine/day	- 7.82	162.1	39	12
nshine	10 mg. thiouracil/day	+ 3.25	132.9	198	10
		Experiment 2			
nky	None	+ 0.25	117.8	-	-
rothy	10 mg. thyroxine/day	- 5.84	121.7	11	18
lfly	10 mg. thyroxine/day	- 6.51	149.3	22	18
ty Morn	20 mg. thiouracil/day	+ 6.20	130.7	254	18
ixie	20 mg. thiouracil/day	+ 5.01	143.1	271	16

Fig. 1 Phosphatase in Cow's Milk (Expt. 1)

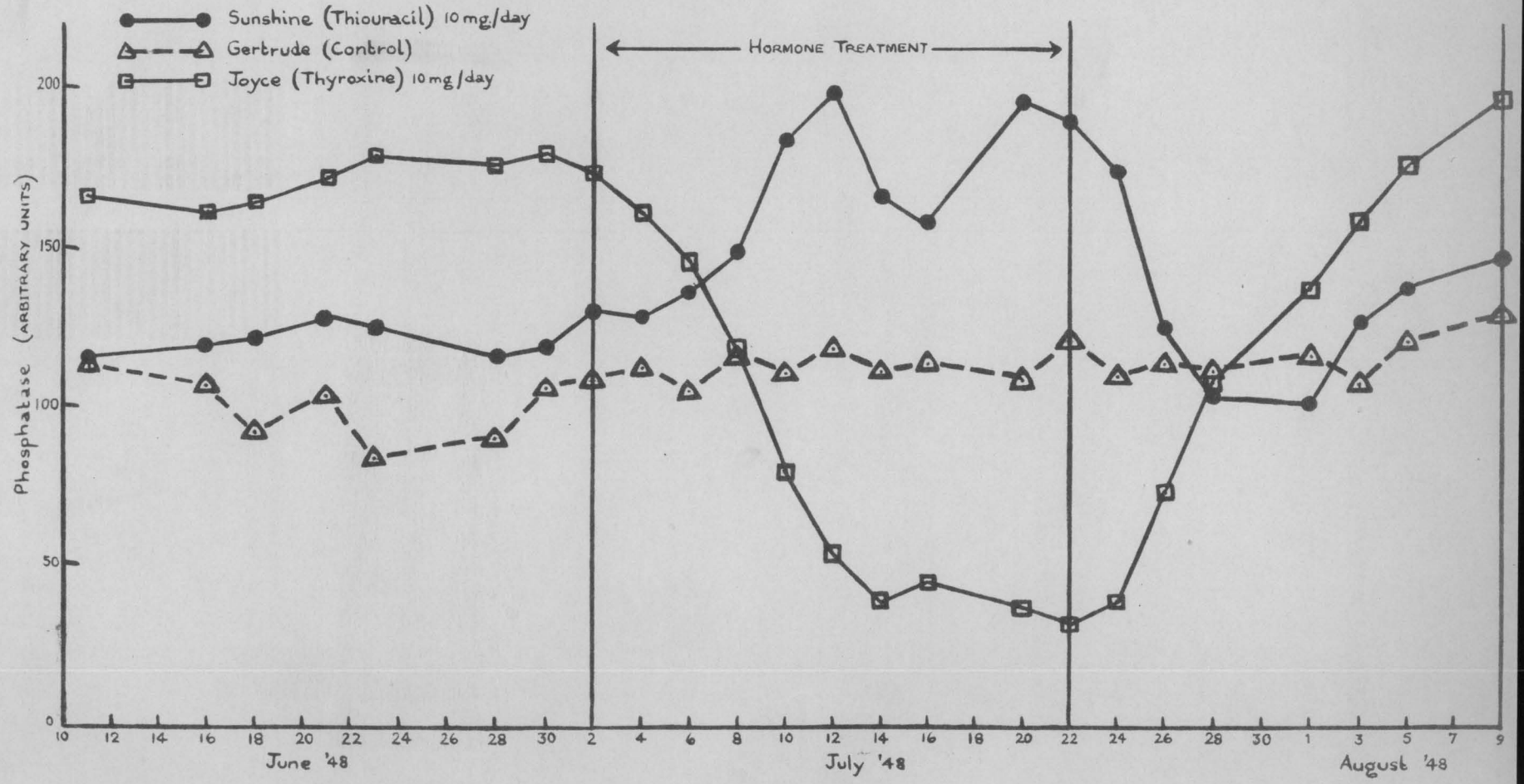
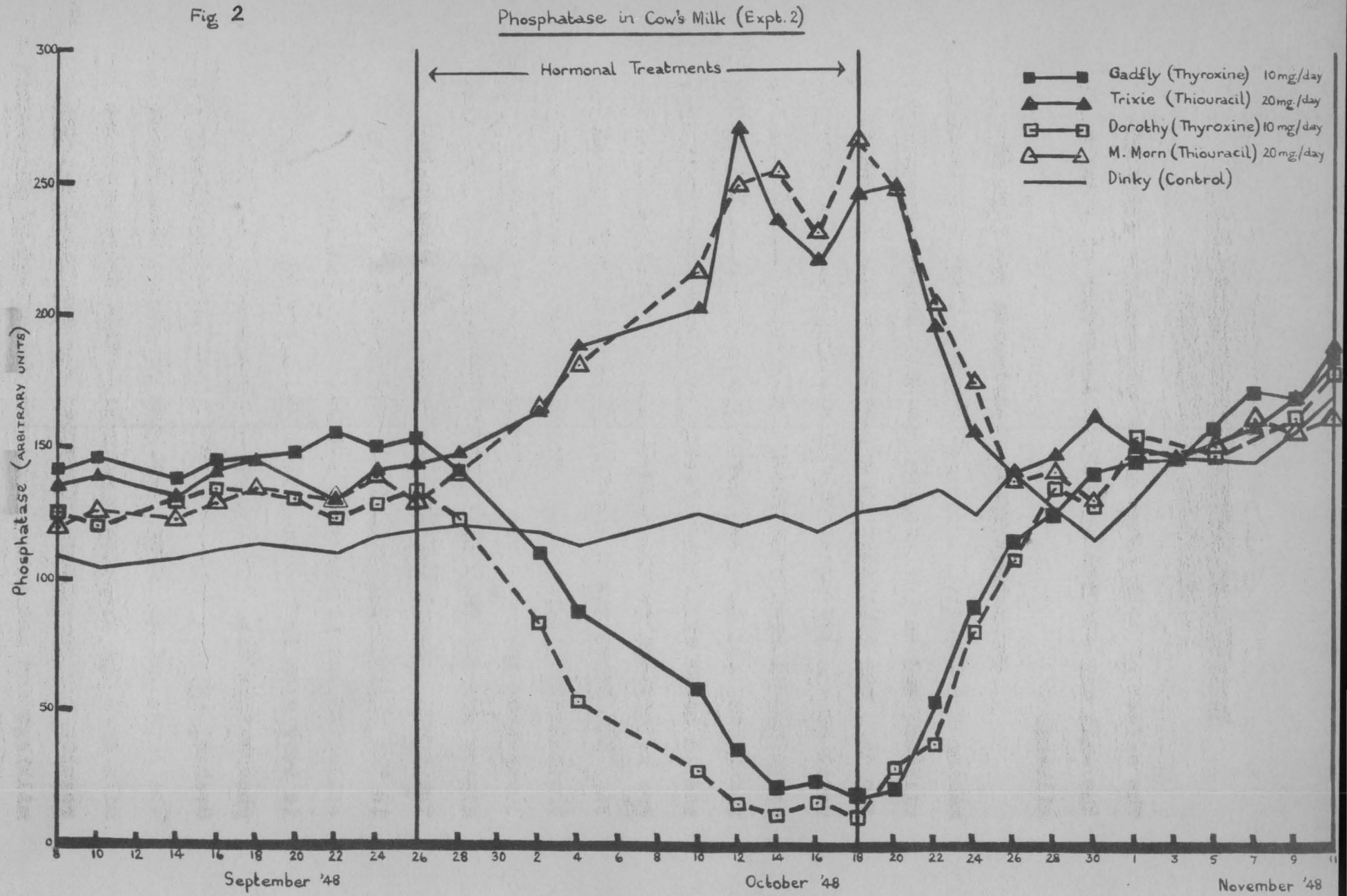


Fig. 2


Phosphatase in Cow's Milk (Expt. 2)
Hormonal Treatments
Gadfly (Thyroxine) 10 mg./day
Trixie (Thiouracil) 20 mg./day
Dorothy (Thyroxine) 10 mg./day
M. Morn (Thiouracil) 20 mg./day
Dinky (Control)
Phosphatase (ARBITRARY UNITS)
300
250
200
150
100
50
0
8 10 12 14 16 18 20 22 24 26 28 30 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 1 3 5 7 9
September '48
October '48
November '48

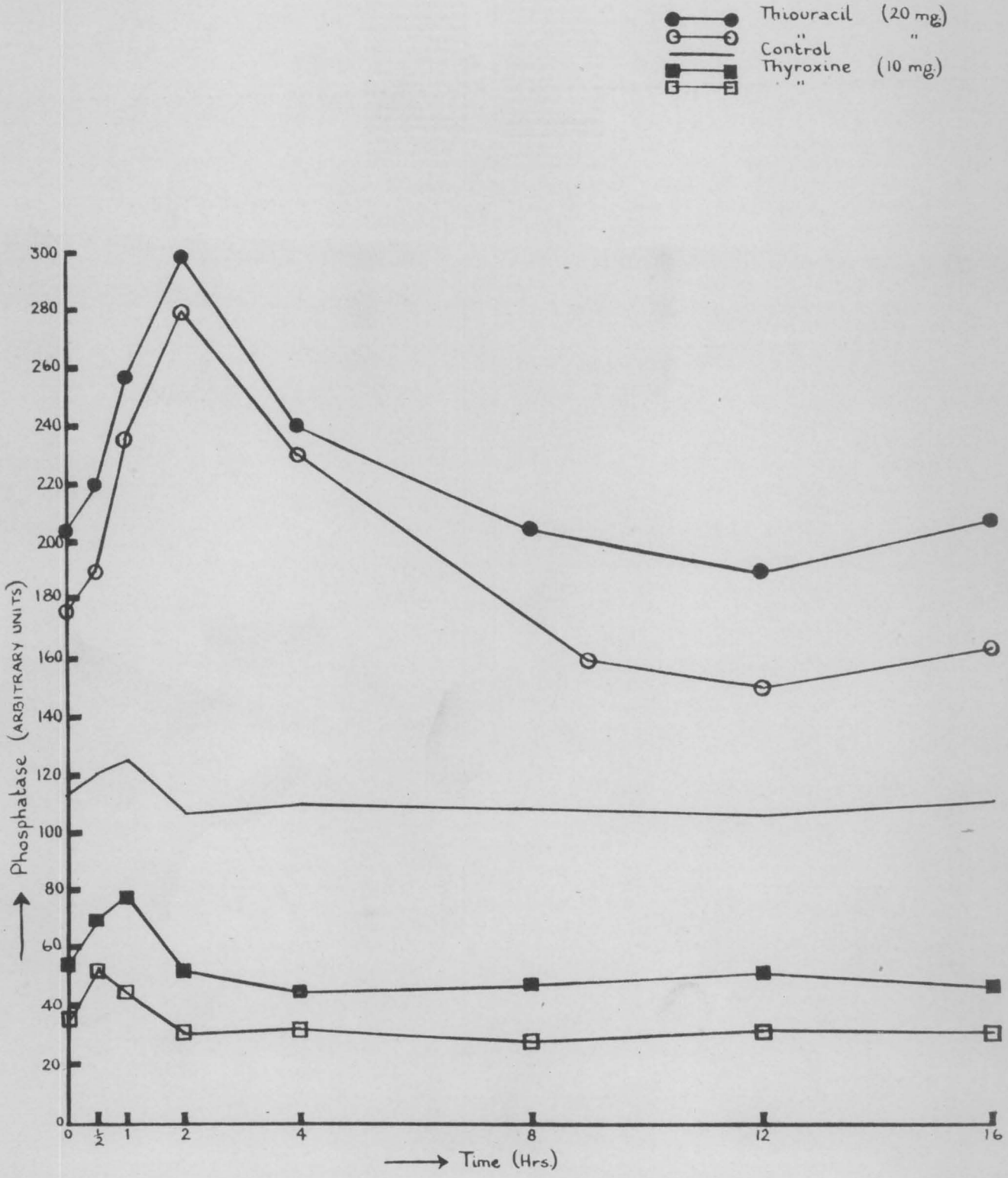
Variation of phosphatase throughout the fifteenth day of treatment

The effect of both drugs on the phosphatase titre of the milk was studied at various intervals of a day's milking.

Throughout these experiments the time of making the injections was immediately after the evening milking, and to investigate the phosphatase throughout the day, the five cows in experiment 2 were milked as usual on the fifteenth day of treatment and the four experimental ones were given their usual injections. Thereafter all the five cows were milked ½, 1, 2, 8, 12 and 16 hours after the previous milking. Phosphatase was determined in the samples with the results shown in Fig. 3. Since the treatments were well advanced the phosphatase titre for the control animal was always lower than that for the thiouracil treated pair and always greater than that for the thyroxine treated pair. There was a small initial increase in the phosphatase in the thyroxine and control cows probably because milk collected, at so short an interval after milking out, is very rich in fat, and cream is known to be richer in phosphatase than the remainder of the milk (Kay & Graham, 1934). A striking effect of this experiment is the large and sustained increase of phosphatase in the milk from the thiouracil animals. This shows that the results for phosphatase obtained for the usual evening milkings did not fully represent the effect of thiouracil on phosphatase. The pronounced maximum which occurred in the milk phosphatase about 2 hr. after milking is

Fig. 3

Changes of Phosphatase throughout the day, from the time of injection on the 15th day of treatment.



probably attributable to ready penetration of the tissues by thiouracil and to the rapidity of elimination of thiouracil by the kidneys (Ely, Olson & Reineke, 1948). The attempt was made to detect thiouracil in the milk by the method of Olson, Ely & Reineke (1947), but, in spite of satisfactory recoveries of thiouracil added to milk in vitro, none could be found. However, this was not surprising because the doses of thiouracil used (about 25 μg./kg. body weight in experiment 1 and 50 μg./kg. body weight in experiment 2) were minute in comparison with the dose of 100 mg. per kg.body weight given subcutaneously and 200 mg. per kg. given by mouth in the experiments of Ely et al. (1948).

General effects of the yield and composition of milk

Thyroxine in these experiments had its expected effect on milk yield. Results for milk yield and for the fat, total N and total P contents of the milk are shown graphically for a control cow in Fig.4a, for two thyroxine treated cows in Figs. 4b & 4c, and for two thiouracil treated cows in Figs. 4d & 4e. The results recorded in Fig. 4b for the thyroxine treated cow, Dorothy, show that the percentage of fat in the milk increased as the milk yield increased. Such responses to thyroxine are often seen in lactating cows. Figs. 4d & 4e contain comparable data from cows treated with thiouracil. The effect of thiouracil in causing the recession of milk yield is notable in the cow Misty Morn (Fig. 4d). The results of the magnitude of the response observed in various

experimentally treated cows will be dealt with in the next chapter along with the proximate composition of the milk. Fig.4 also shows the effects of the drugs on the total N and total P content of milk. It will be observed that there is a marked contrast between the constancy of total P in the milk of the untreated cow, Dinky (Fig.4a) and its increase in the thyroxine treated cows, Dorothy & Gadfly (Fig. 4b & 4c). Equally contrasting is the decrease of total P produced by thiouracil (Fig.4d & 4c). Further contrasts can be noticed by comparing the total P with the total N. It will be noticed that in spite of the increase in total P in the milk of the thyroxine cows and the decrease in that of the thiouracil cows, there was no consistent change in the total N when the results were expressed on a fat-free basis. Also, in the whole milk, no regular change in nitrogen concentration was noticeable but the values tended to be slightly lower when a higher phosphorus concentration occurred in the milk of the thyroxine cows.

Some typical results of N:P ratio in the whole milk are recorded in Table 4. For each animal values before treatment have been compared with those obtained at the time of maximum response to the drugs. For the control cows the ratios during corresponding periods are shown for comparison. It will be observed that there was a negligibly small increase in the ratio in the two control cows as lactation progressed from period 1 to period 2, but the increase resulting from thiouracil treatment was much larger, and there was a

big decrease in the ratio as a result of thyroxine treatment. These effects can plainly be seen in Table 4 but can also be inferred from the results for total nitrogen and total P shown in Fig.4.

Table 4. The ratio of nitrogen to phosphorus in cows' milk at time of maximal response* to thyroxine or thiouracil

Cow	Treatment in Period 2	Period	Total N (mg./100 g.)	Total P (mg./100 g.)	N:P
Gertrude	None	1	599	88	6.8
		2	615	88	7.0
Joyce	Thyroxine	1	587	92	6.4
		2	549	99	5.5
Sunshine	Thiouracil	1	525	85	6.2
		2	547	72	7.6
Dinky	None	1	550	89	6.2
		2	564	88	6.4
Dorothy	Thyroxine	1	582	86	6.8
		2	577	98	5.9
Gadfly	Thyroxine	1	553	90	6.1
		2	519	98	5.3
Misty Morn	Thiouracil	1	499	91	5.5
		2	496	80	6.2
Trixie	Thiouracil	1	573	99	5.8
		2	612	89	6.9

* Maximal response denotes the smallest value of N:P produced by thyroxine and the largest value produced by thiouracil.

Partition of nitrogen

The effects of the drugs on the partition of nitrogen were investigated. The results of total N and the percentages of nitrogen in the forms of casein, albumin, globulin, proteose peptone and non-protein

Fig. 4a DINKY

The Composition of Cow's Milk. (Expt. 2)

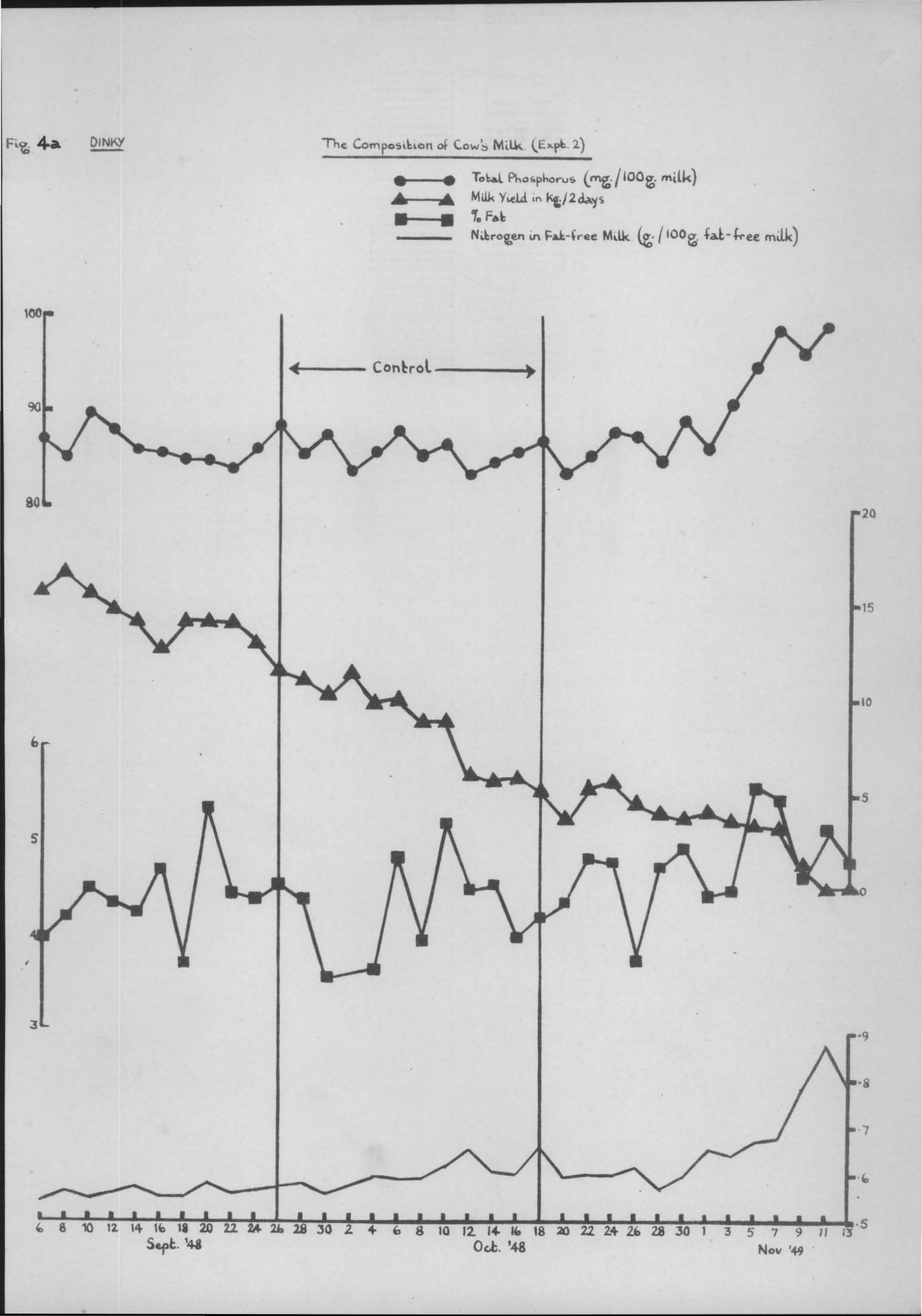
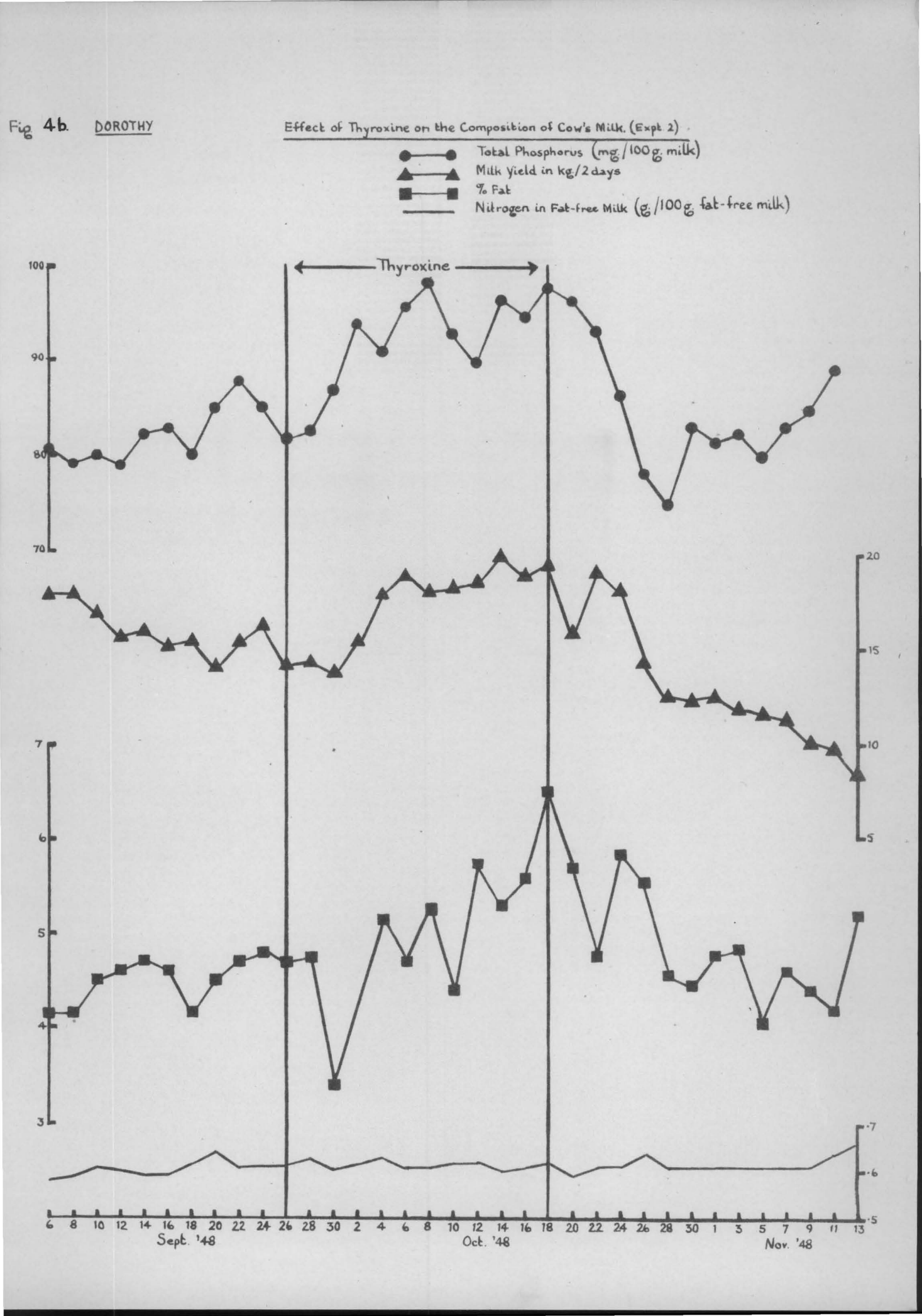
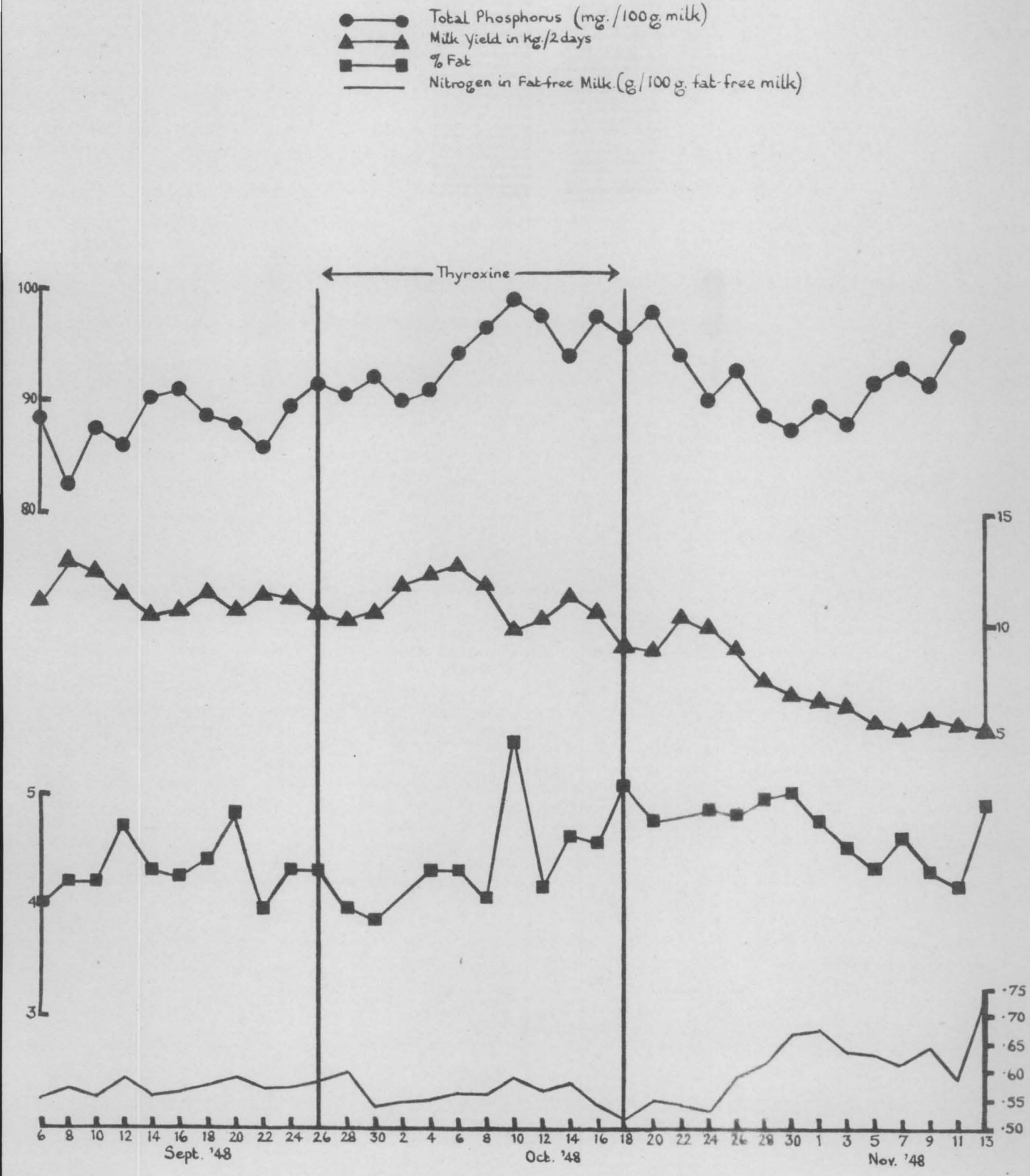


Fig 4b. DOROTHY

Effect of Thyroxine on the Composition of Cow's Milk. (Expt 2)



Effect of Thyroxine on the Composition of Cow's Milk (Expt 2)



4d MISTY MORN

Effect of Thiouracil on the Composition of Cow's Milk. (Expt. 2)

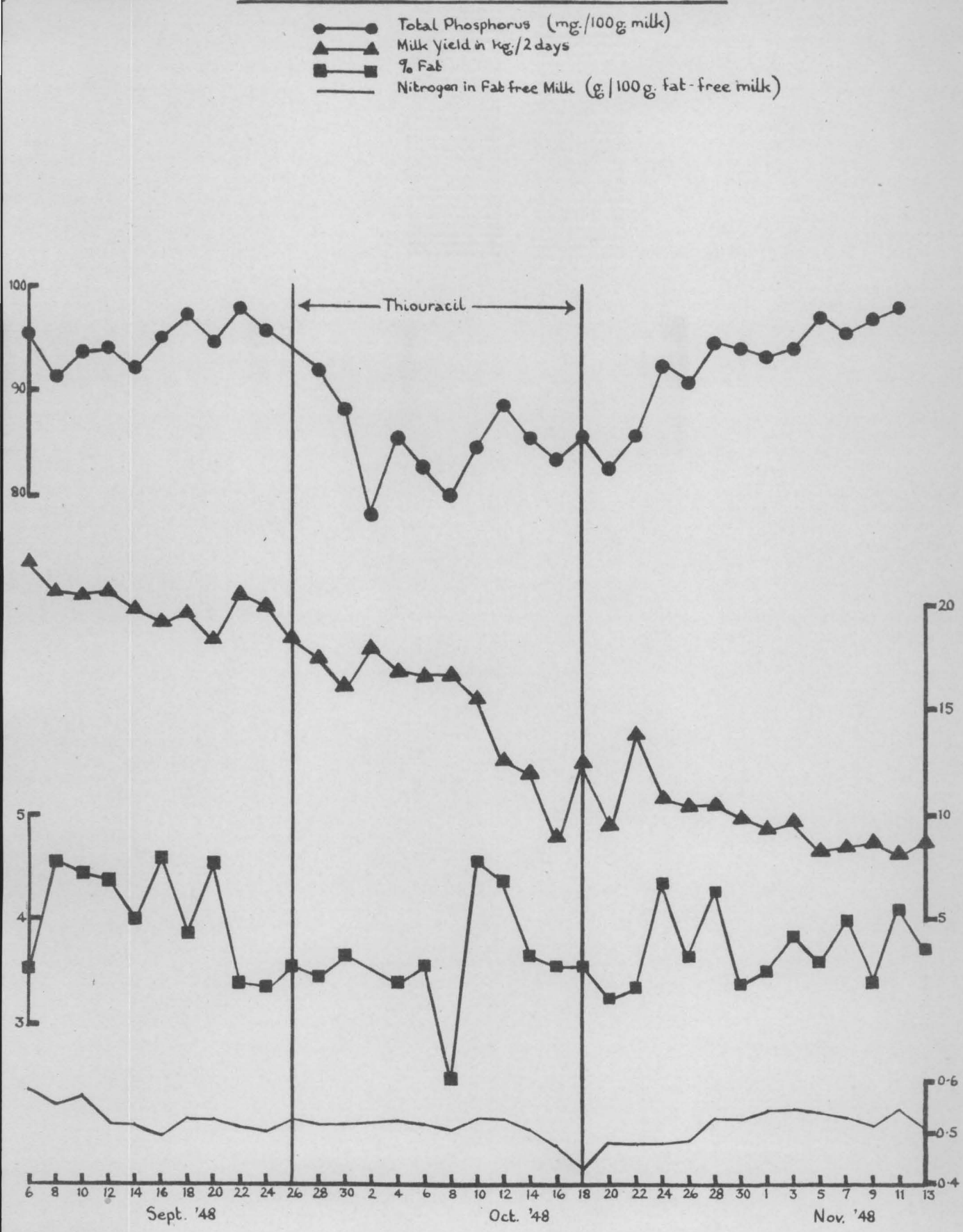
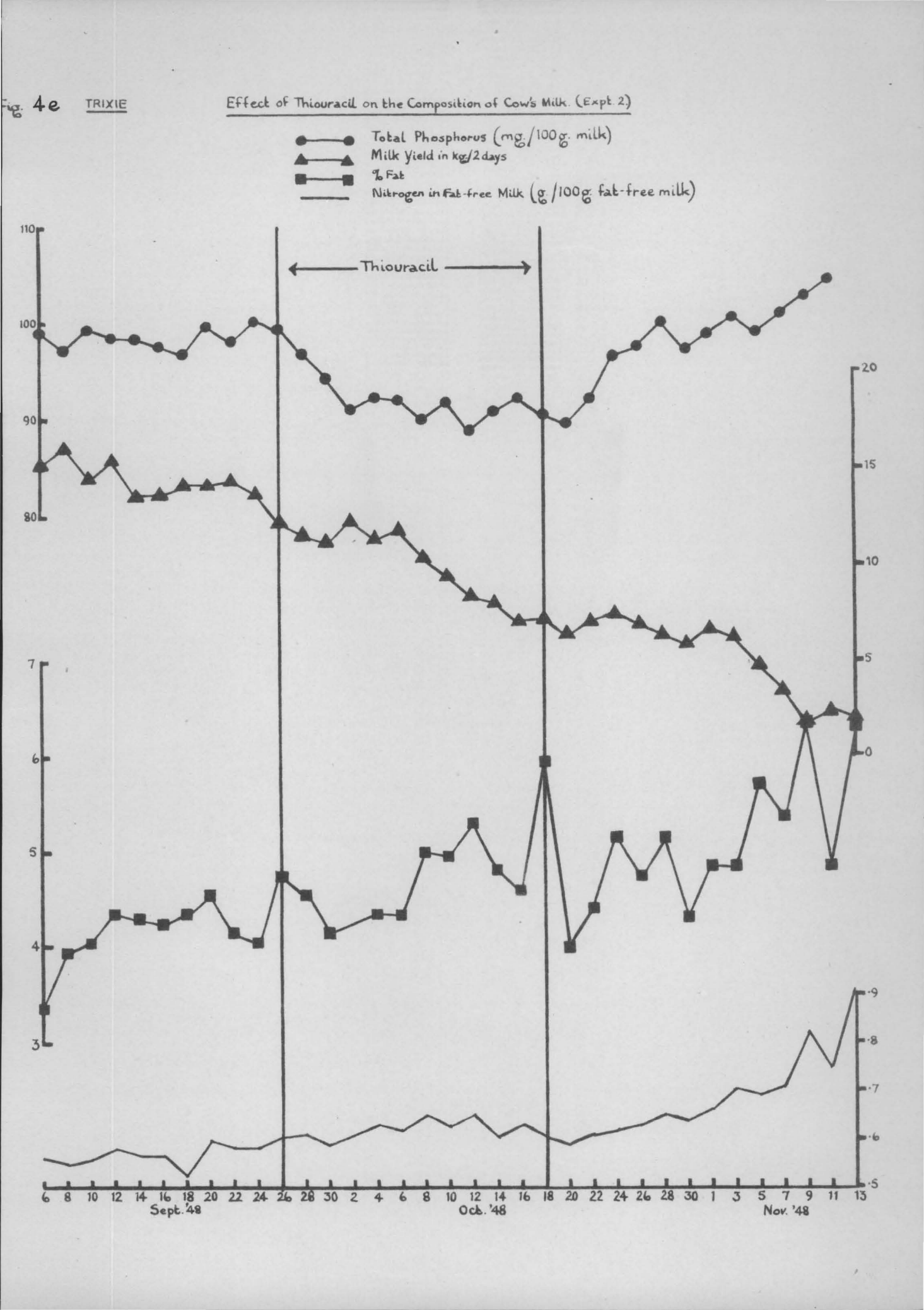


Fig. 4e TRIXIE

Effect of Thiouracil on the Composition of Cow's Milk. (Expt. 2)



nitrogen are recorded in Table 5. The standard errors of each fraction are also shown in the table. Neither thyroxine nor thiouracil affected the partition of nitrogen. This finding agrees with those of Van Landingham, Hyatt & Weakley (1946) and Booth, Elvehjem & Hart (1947) who partitioned the nitrogen in milk of cows receiving iodinated casein. The lack of change in the partition of nitrogen is in marked contrast to the simultaneous effects on phosphorus partition which is described below. The results of the nitrogen partition are, however, of considerable interest when the changes are considered from the point of view of advancing lactation. It can be seen that in all the cows as lactation progressed the casein number (percentage of total N in the form of casein) progressively decreased. In contrast there was a large increase in the albumin fraction with all the animals during the last period of the experiment, when the cows were rapidly drying off. There was an equally dramatic increase in the globulin fraction during the corresponding period (Period 3). The proteose-peptone fraction also became greater as lactation advanced. These effects occurred in all the cows irrespective of the treatments.

Partition of phosphorus

The partition of phosphorus in milk is shown in Fig.5. Graphs of the results are recorded for two control cows (Figs. 5a & 5b), three thyroxine treated cows (Figs.5c, 5d & 5e) and three thiouracil treated cows (Figs. 5f, 5g, & 5h). The range of

numerical values during the treatment period (Period 2), pretreatment period (Period 1) and the recovery period (Period 3) are recorded in Table 6. The mean values of total P and its partition between inorganic P, ester P, lipid P and casein P are shown in Table 7. In this table the results of each fraction are expressed as percentages of total P. The average phosphatase titre is also recorded in the table for comparison. The results in Tables 6 & 7 confirm the earlier observation of Owen (1948b) who found a notable increase of total P in the milk of thyroxine treated cows. They also record the new observation that thiouracil has a marked effect but in the reverse direction to that produced by thyroxine. Thus thiouracil markedly reduces the percentage of total P in the milk. The control animals show that there is a natural tendency for the milk to become richer in phosphorus as lactation advances. That thyroxine enhances while thiouracil inhibits this tendency is also shown in Tables 6 & 7.

Comparison of the phosphorus partition data recorded in Table 7 with Fig.5 in which individual 2-day data are graphed, serves to show that the changes in partition which resulted from hormonal treatments are not shown in their full magnitude by the averages in Table 7. The most notable change recorded in both Fig.5 and Table 7 occurred in the ester-phosphorus which was markedly increased by thyroxine and markedly decreased by thiouracil. The change which occurred in the lipid phosphorus can be seen in Table 6 and also

in Fig. 5. It changed in the same way as the ester-phosphorus but not so markedly. It will be noticed that in the animals, Dorothy and Gadfly, which were treated with thyroxine, the maximum lipid phosphorus values were much higher in the treatment and post-treatment periods than in the pre-treatment period. The maximum values in the recovery period (Period 3) were obtained during the day immediately after cessation of treatment. The same sort of changes occurred also in the casein-P but do not show up to advantage in Table 7. In Fig.5 and in Table 6 the effects of thyroxine and thiouracil on casein-P are evident. Table 6 shows that the maximum value was increased in all the three thyroxine cows. In the thiouracil cows the minimum values for ester-P, lipid-P and casein-P were reduced during the treatment period. Casein in milk became for a time richer in phosphorus during thyroxine treatment and poorer in phosphorus during thiouracil treatment. Tables 6 & 7 show that changes of ester-P, lipid-P and casein-P produced by treatment with either thyroxine or thiouracil were in the opposite direction to the change simultaneously produced in phosphatase. This is referred to again later.

Table 7 shows that the increase of total phosphorus by thyroxine tended to conceal a decrease of inorganic phosphate and also that the decrease of total phosphorus by thiouracil tended to conceal an increase in inorganic phosphate. Thus inorganic phosphate differed from all the other fractions

investigated in changing under either treatment in the same direction as did phosphatase. Comparison of Fig. 2 with Fig.5 indicated how closely phosphatase and ester-P were correlated. Accordingly coefficients of correlation of phosphatase with various phosphorus fractions were calculated. These coefficients are recorded in Tables 8 & 9. They show that during either treatment phosphatase was closely correlated negatively with ester and lipid phosphorus and positively with inorganic phosphorus. Table 8 shows that the correlation between ester phosphorus and phosphatase was large in the untreated cows and in the treated cows during the pre-treatment period. The same generalisation does not, however, apply to the lipid phosphorus (Table 9) which in the control cows and in the pre-treatment periods of the treated cows was not significantly correlated with phosphatase at a 5% level of significance. The correlations between ester-P and phosphatase were found to be significantly increased by treatment with thyroxine or thiouracil.

Fisher's Z values are also recorded in Table 8 and the difference between a control and a treated cow was always found to be significant. The negative correlation of ester-P and phosphatase are shown in Fig.6 for a control cow (Fig.6a), for a thyroxine cow (Fig.6b) and a thiouracil cow (Fig.6c). Curves have been fitted by the method of least squares. The analysis of variance showing significant departure from linearity is shown in Table 10 for a thyroxine

treated cow. There is thus a curvilinear relationship between ester-P and phosphatase similar to that reported in Chapter IV for the relationship between phosphatase and ester-P in human milk.

The positive correlation between inorganic phosphorus and phosphatase are shown in Fig.7 for a control cow (Fig.7a), a thyroxine treated cow (Fig.7b) and a thiouracil treated cow (Fig.7c). This positive correlation was also significantly increased by treatment with either drug. The negative correlation between lipid-P and phosphatase is recorded in Fig.8 for a thyroxine cow (Fig.8a) and also for a thiouracil cow (Fig.8b). For these diagrams only values obtained during the treatment and post-treatment periods were used, since the correlation did not hold during the pre-treatment period. The correlation diagram for a control cow has also been omitted for the same reason.

Fig. 5a. GERTRUDE

Partition of Phosphorus in Milk. (Expt. I)

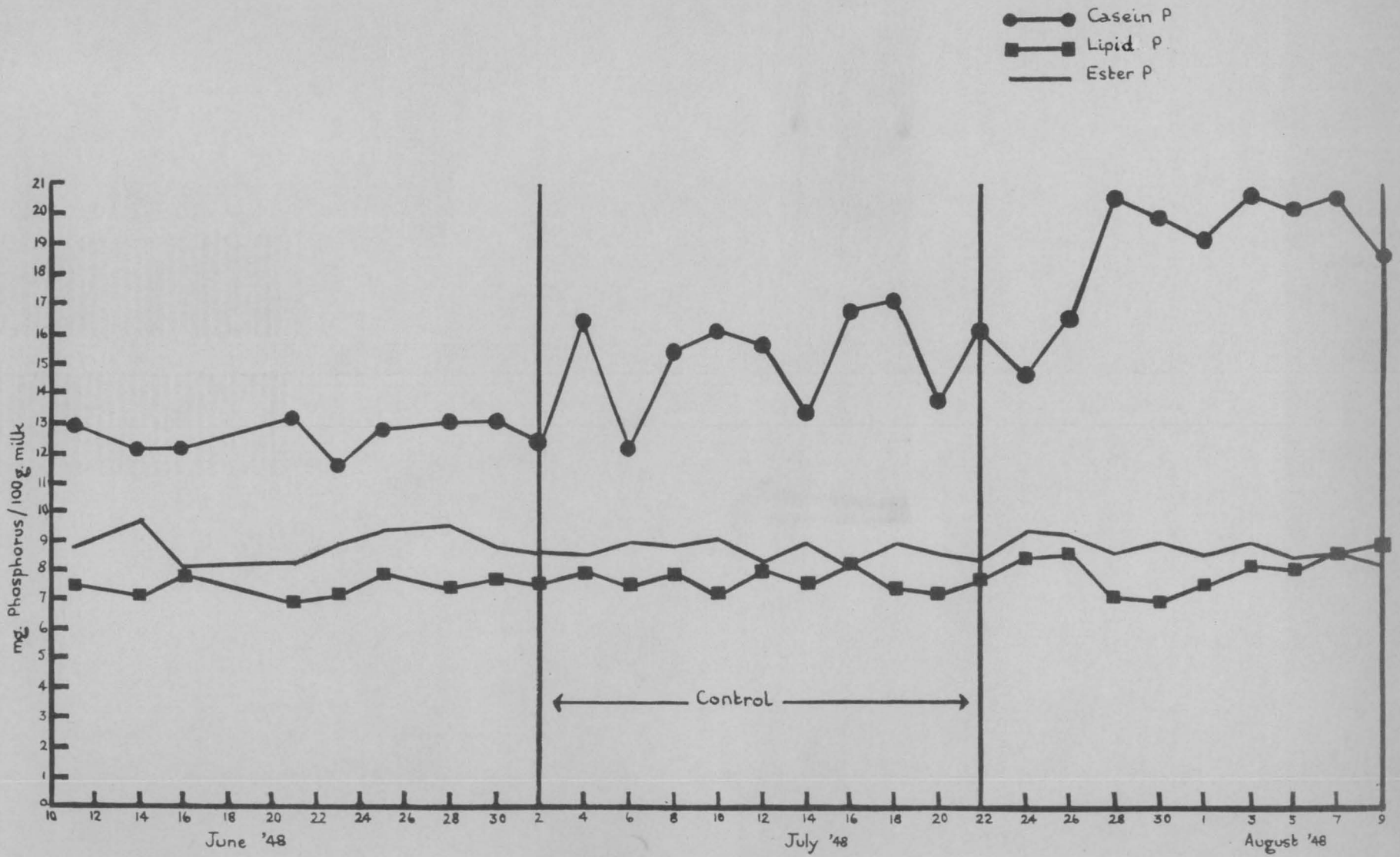


Fig. 5b DINKY

Partition of Phosphorus in Milk. (Expt. 2)

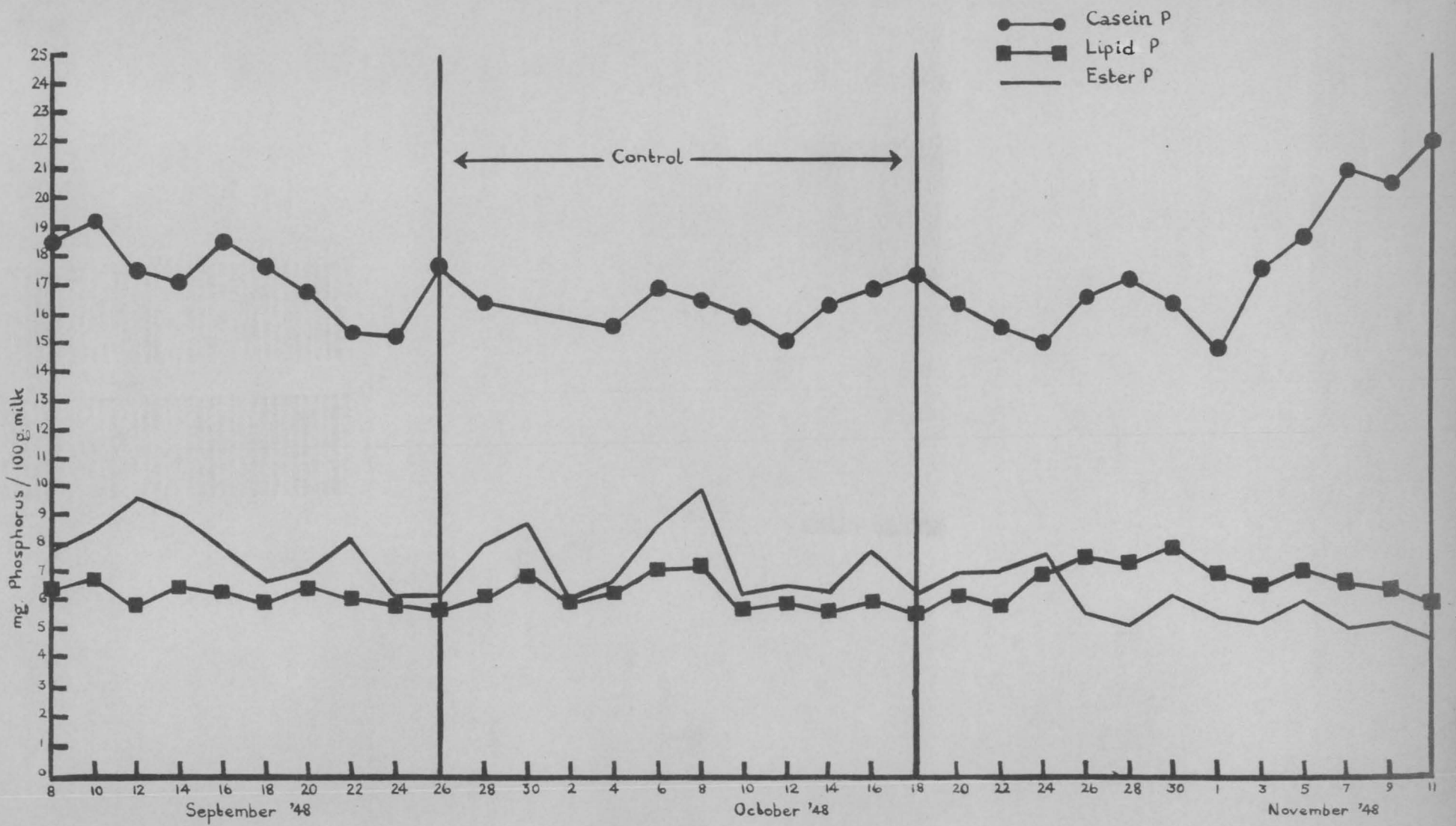


Fig. 5c. JOYCE

Effect of Thyroxine on the Partition of Phosphorus in Milk. (Expt. I)

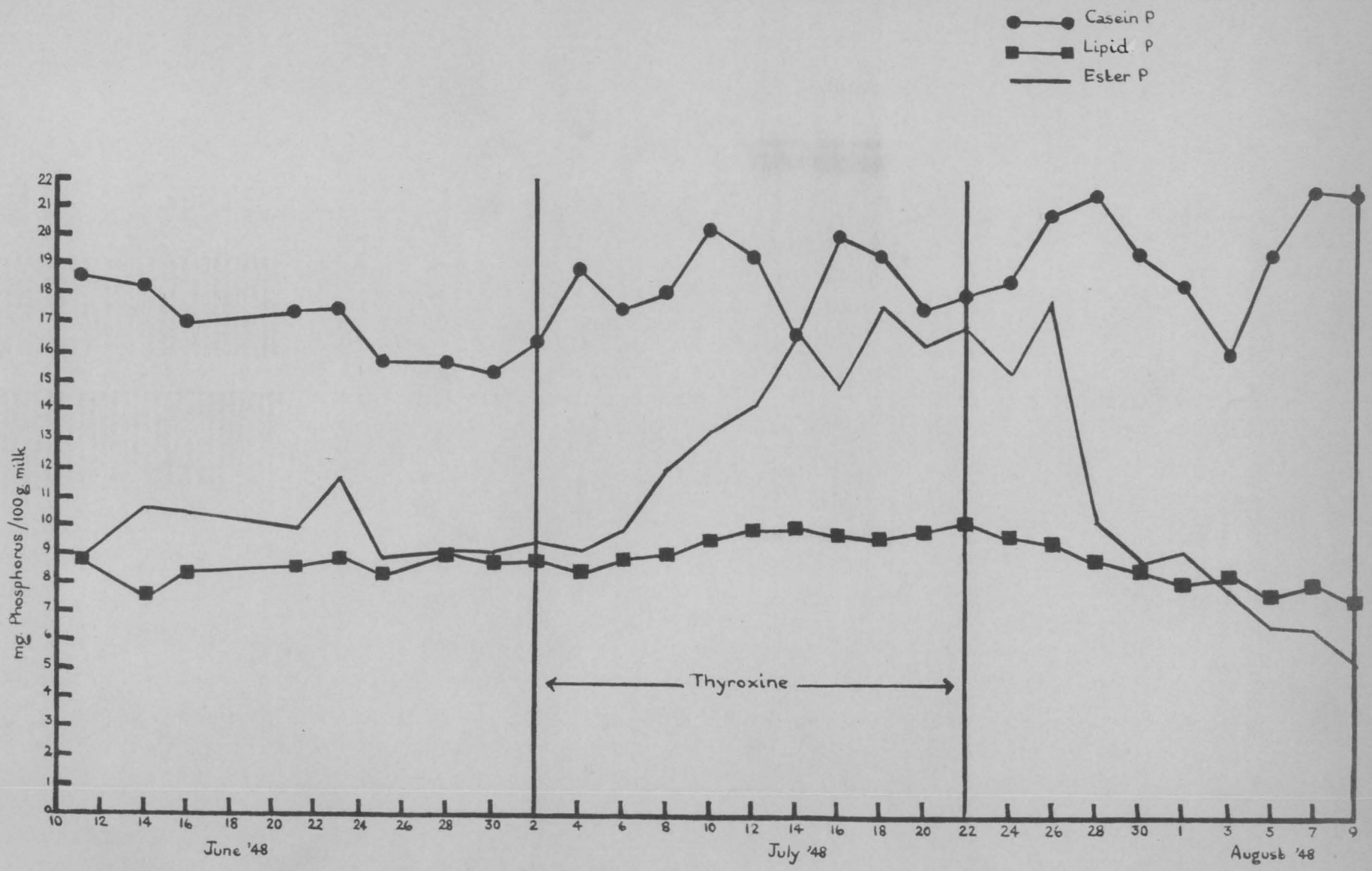


Fig. 5d DOROTHY

Effect of Thyroxine on the Partition of Phosphorus in Milk. (Expt 2)

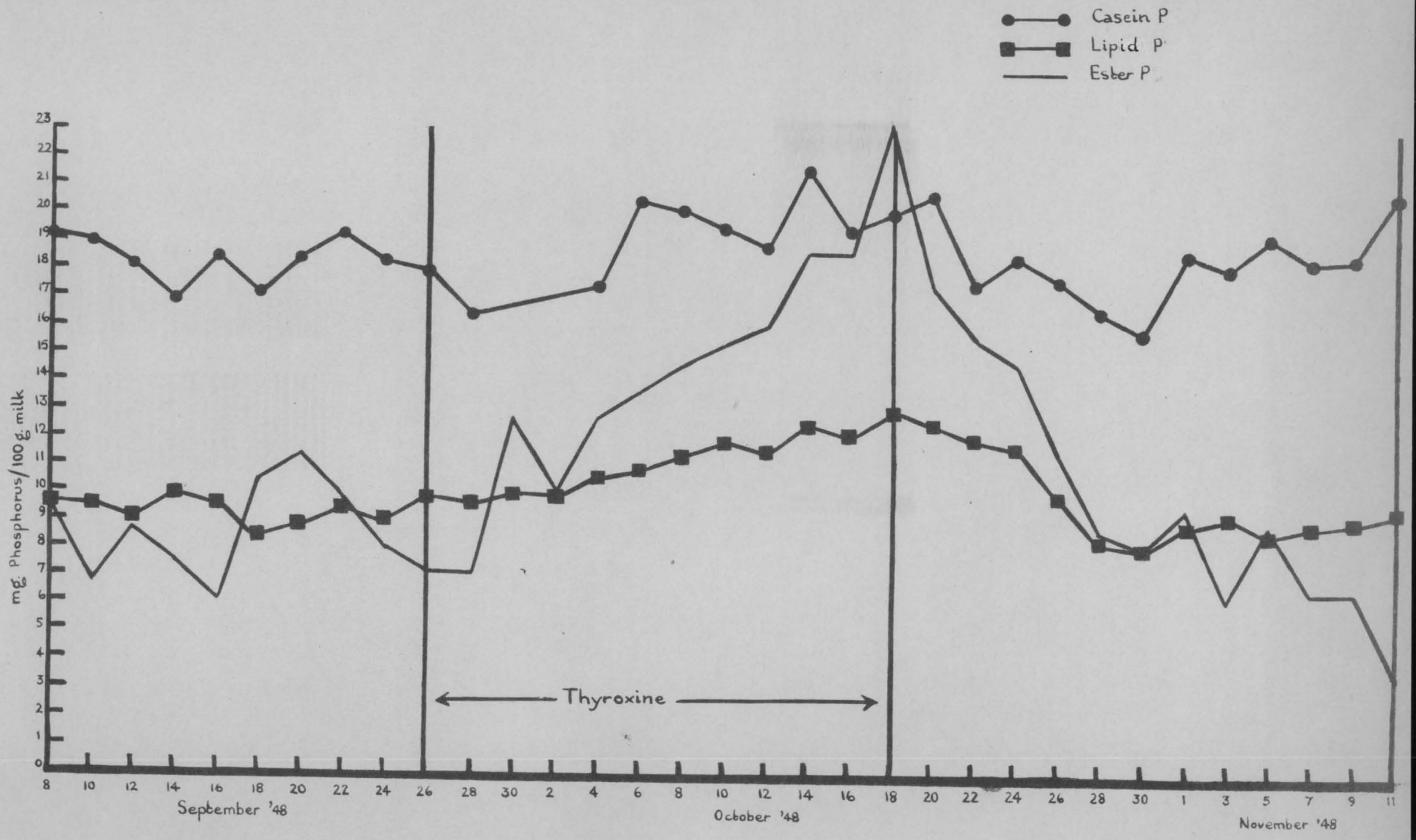


Fig. 5e. GADFLY

Effect of Thyroxine on the Partition of Phosphorus in Milk (Expt. 2)

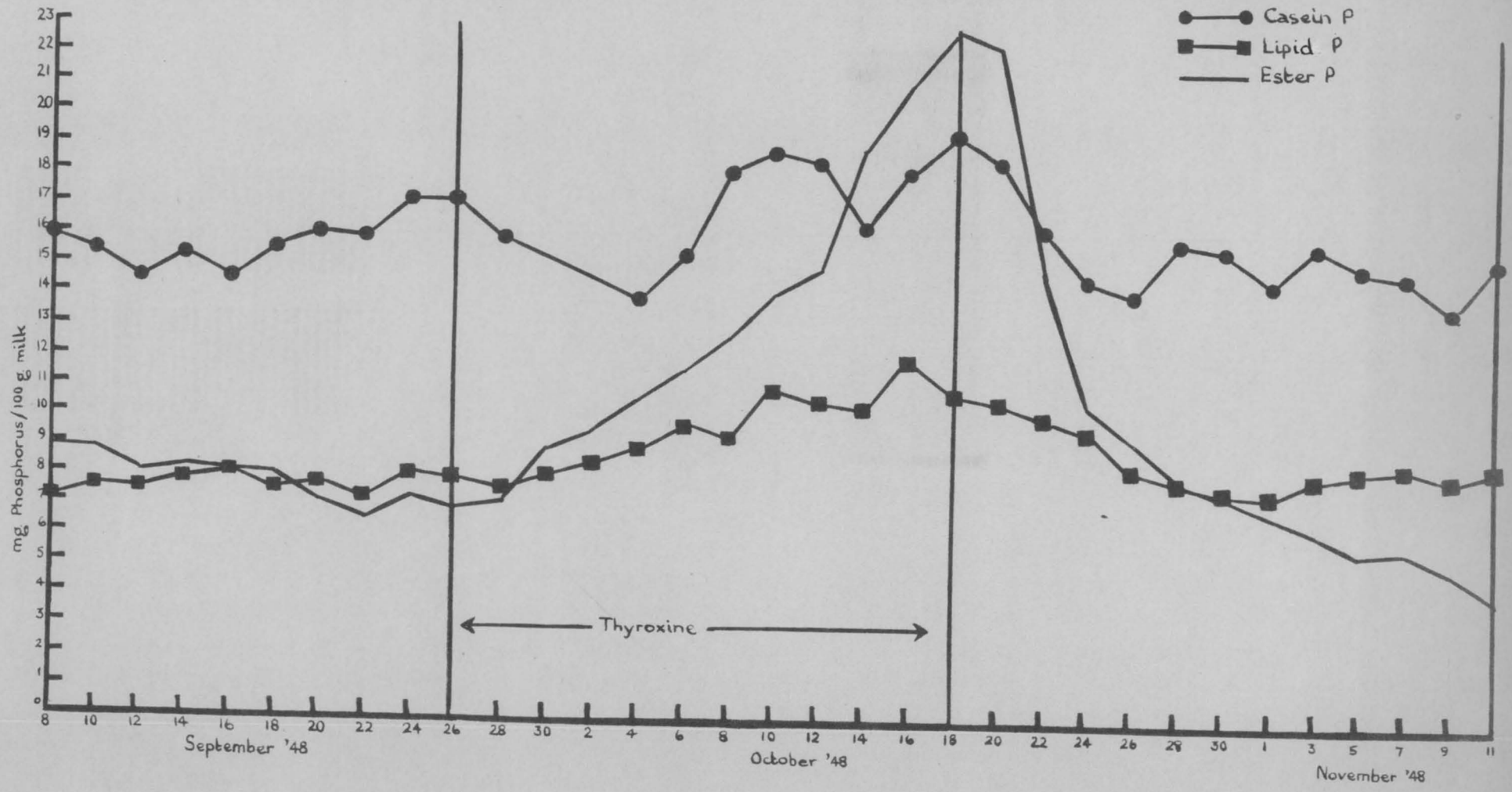


Fig 5f. SUNSHINE

Effect of Thiouracil on the Partition of Phosphorus in Milk. (Expt. I)

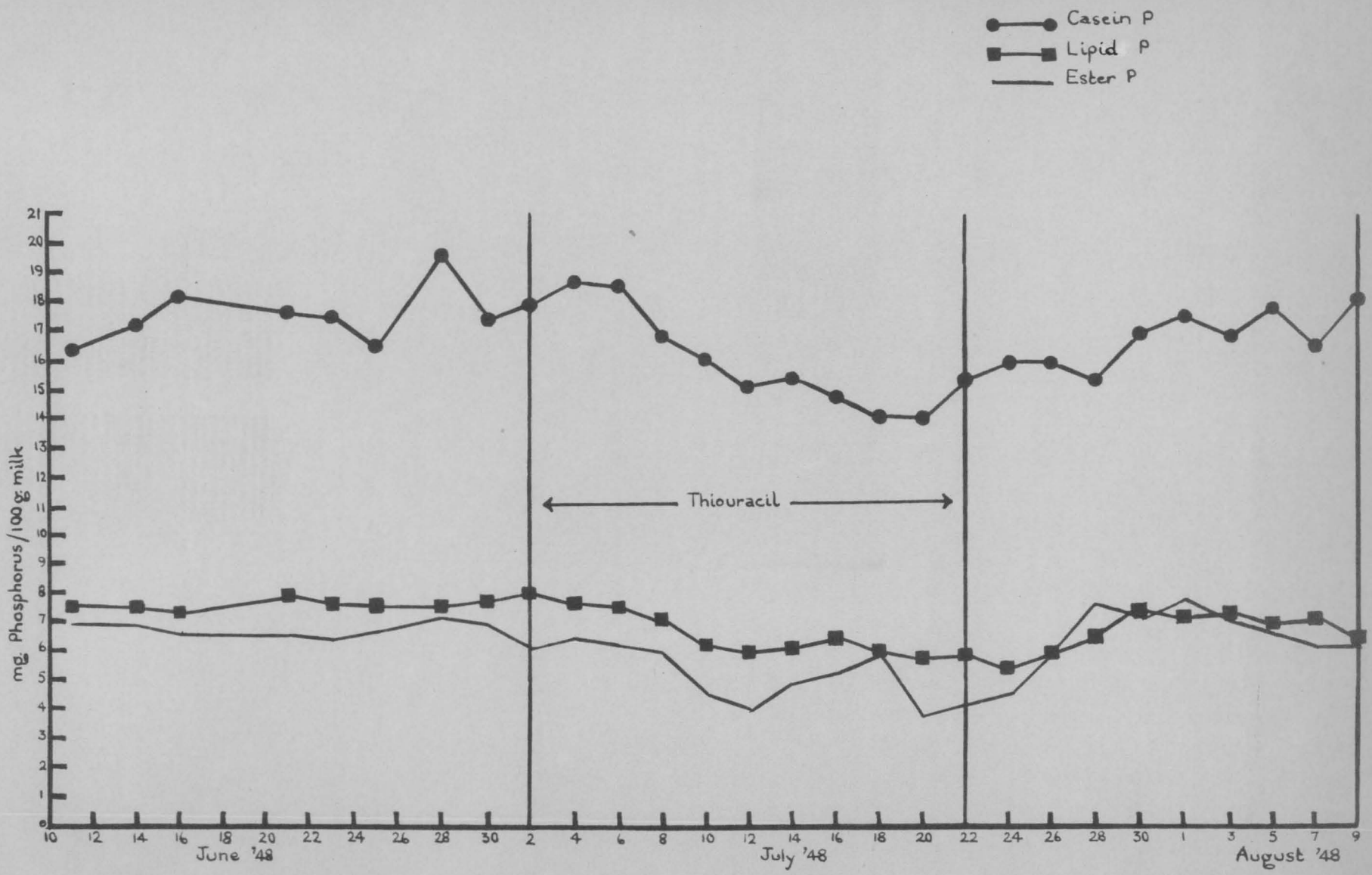


Fig. 5g. MISTY MORN

Effect of Thiouracil on the Partition of Phosphorus in Milk. (Expt. 2)

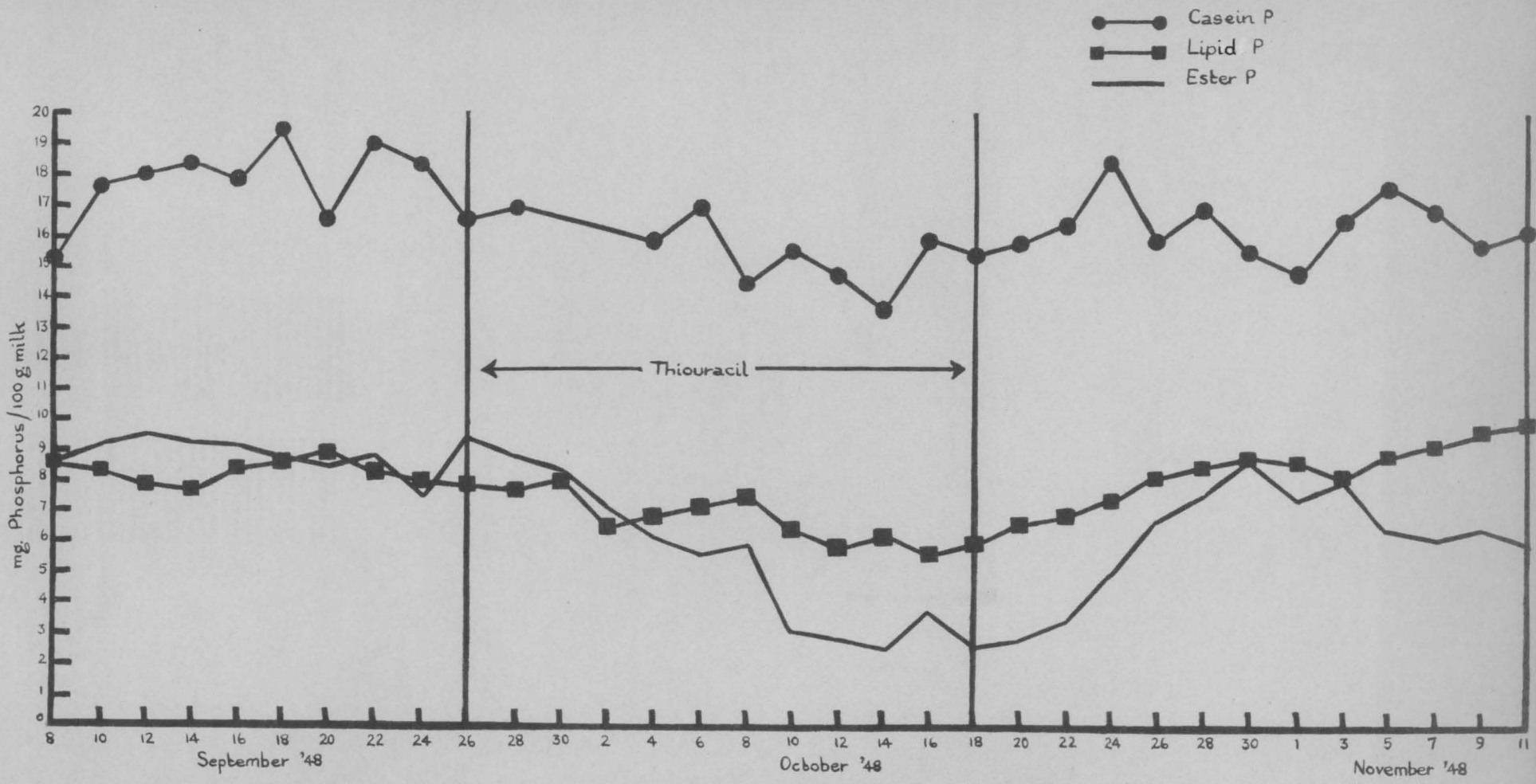


Fig. 5h. TRIXIE

Effect of Thiouracil on the Partition of Phosphorus in Milk (Expt. 2)

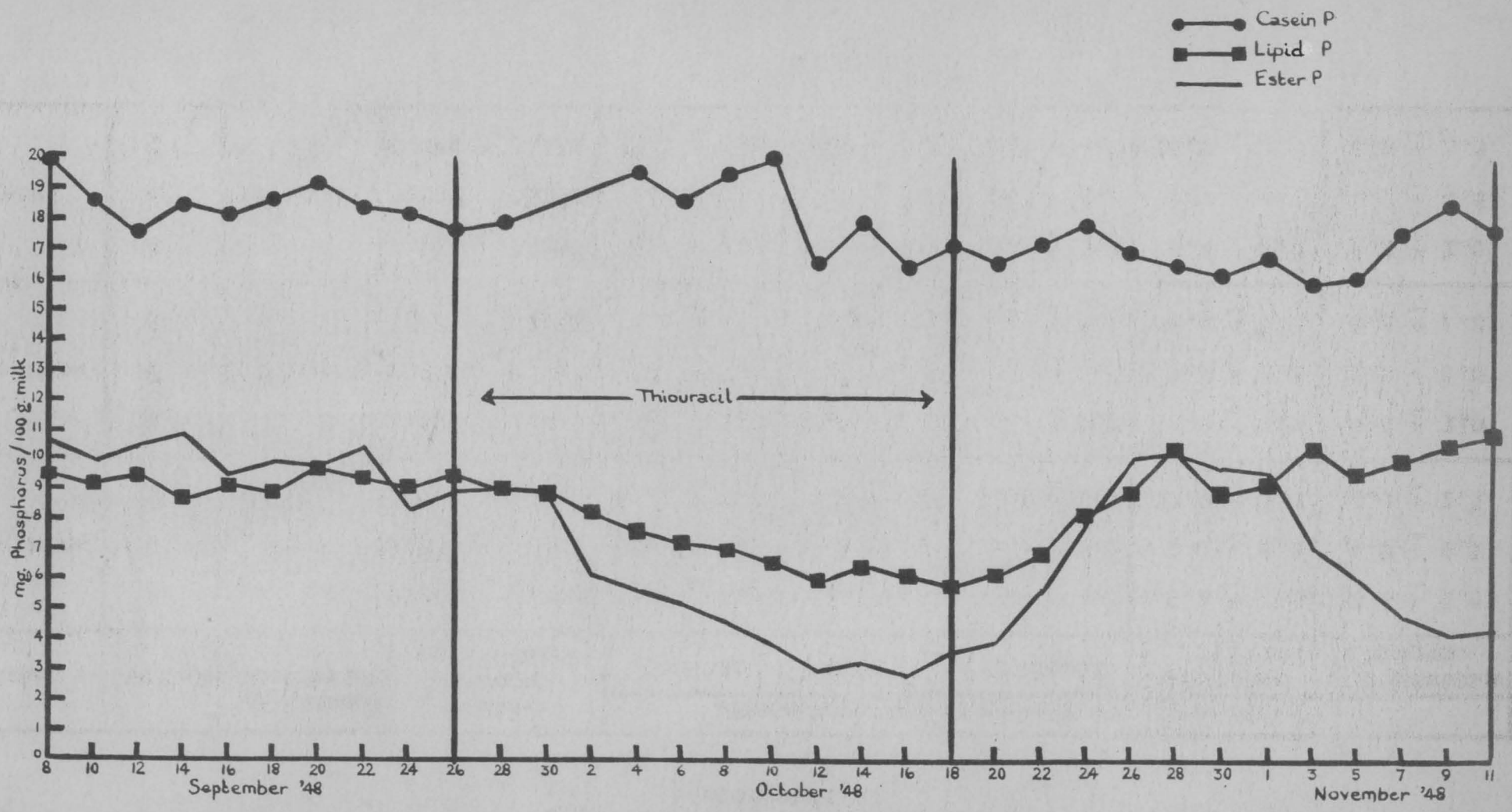


Table 5. Partition of nitrogen in the milk of normal cows and of cows receiving thyroxine or thiouracil

(Experiment 1)

Cow	Period	Treatment in Period 2	Total nitrogen (mg./100g.)	Percentage of nitrogen in the form of :-				
				Casein	Albumin	Globulin	Proteose peptone	Non-protein nitrogen
Gertrude	1	None	0.604 ± 0.045	74.6 ± 4.5	9.0 ± 2.2	5.4 ± 2.0	6.8 ± 1.6	3.6 ± 1.0
	2		0.622 ± 0.061	68.5 ± 3.4	11.9 ± 3.2	9.1 ± 3.0	9.0 ± 5.9	4.7 ± 0.7
	3		0.688 ± 0.052	64.4 ± 5.0	3.9 ± 0.7	14.1 ± 3.6	13.9 ± 3.6	4.2 ± 1.1
Sunshine	1	Thiouracil	0.518 ± 0.054	78.3 ± 3.3	10.3 ± 3.5	4.1 ± 5.3	5.6 ± 0.9	3.8 ± 1.0
	2		0.519 ± 0.055	76.0 ± 2.4	9.7 ± 2.3	4.3 ± 2.6	7.3 ± 3.1	5.0 ± 0.5
	3		0.537 ± 0.051	71.0 ± 4.5	5.4 ± 1.4	9.3 ± 4.1	8.4 ± 3.1	5.8 ± 1.2
Joyce	1	Thyroxine	0.592 ± 0.057	74.0 ± 4.1	10.1 ± 2.8	5.8 ± 3.9	6.4 ± 1.2	3.8 ± 1.0
	2		0.607 ± 0.036	71.5 ± 2.6	12.9 ± 3.9	5.4 ± 3.6	9.9 ± 4.7	4.7 ± 0.5
	3		0.769 ± 0.164	63.7 ± 8.6	4.6 ± 0.5	13.7 ± 4.3	13.7 ± 4.3	3.9 ± 1.0

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Table 6. Partition of phosphorus in the milk of normal cows and cows receiving thyroxine or thiouracil

(Range of values)

Cows	Period	mg./100 g. milk					Phosphatase (arbitrary units)
		Total P	Inorganic-P	Ester P	Lipid-P	Casein-P	
ertrude	1	80.5- 84.2	52.0-55.0	8.1- 9.7	6.9- 7.8	11.5-13.2	84-120
ontrol)	2	80.1- 92.4	51.5-59.2	8.2- 9.1	7.2- 8.2	12.2-17.1	105-121
	3	86.9- 99.4	54.6-63.2	8.1- 9.3	6.9- 7.1	14.6-22.1	108-129
oyce	1	88.5- 95.9	53.9-59.5	8.9-11.6	7.5- 8.9	15.3-18.7	162-179
hyrox-	2	92.8- 99.4	47.2-61.0	9.2-17.6	8.4-10.2	16.7-20.3	32-161
ine)	3	86.1- 99.7	50.1-62.9	5.3-15.4	7.5- 9.7	16.1-21.7	39-196
unshine	1	80.8- 90.9	48.8-58.8	6.1- 7.2	7.0- 8.0	16.5-19.6	116-130
hioura-	2	72.1- 85.9	46.0-55.0	3.7- 6.4	5.7- 7.7	14.0-18.7	128-198
cil)	3	76.9- 90.6	47.5-58.1	4.5- 7.8	5.4- 7.4	15.3-21.0	102-174
inky	1	84.1- 89.8	53.1-59.1	6.1- 9.6	5.7- 6.7	15.3-19.3	105-119
ontrol)	2	83.6- 88.4	53.6-58.9	6.0- 9.9	5.5- 7.2	15.6-17.4	114-126
	3	83.6- 99.4	54.5-64.4	4.6- 7.6	5.8- 7.8	14.8-21.8	116-169
orothy	1	79.4- 88.4	41.2-49.9	6.2-11.4	8.5- 9.9	16.8-19.2	122-135
hyrox-	2	83.0- 98.3	42.0-52.5	7.2-23.1	9.6-12.9	16.4-21.5	11-123
ine)	3	80.1-96.5	40.9-56.1	3.6-17.3	8.1-12.5	15.8-20.6	29-178
adfly	1	82.9- 91.7	51.2-60.0	6.6- 8.9	7.1- 8.1	14.2-17.2	138-156
hyrox-	2	90.0- 99.2	42.8-62.0	7.1-22.9	7.6-11.9	13.9-19.4	19-141
ine)	3	88.1- 98.1	47.0-67.3	4.2-22.3	7.6-10.6	13.8-18.5	22-185
isty Morn	1	91.3- 97.8	57.3-62.5	7.5- 9.9	7.8- 8.9	15.3-19.1	120-139
hioura-	2	78.5- 92.2	50.4-64.4	2.5- 8.8	6.1- 8.0	13.7-16.9	141-266
cil)	3	82.7- 98.3	58.4-66.8	2.8- 8.6	6.6- 9.9	14.8-18.5	131-248
rixie	1	97.3-100.4	58.4-64.0	8.3-10.8	8.7- 9.7	17.5-19.9	131-145
hioura-	2	96.8- 88.9	59.0-65.4	2.8- 9.1	5.7- 9.0	16.4-20.3	148-271
cil)	3	89.5-104.6	61.5-71.5	3.8-10.5	6.2-10.9	15.9-18.5	142-249

Table 7. Partition of phosphorus in the milk of normal cows and cows receiving thyroxine or thiouracil

Cow	Period	Total phosphorus (mg./100g. milk)	Percentage of total phosphorus as: Inorganic	Ester	Lipid	Casein	Phosphatase (arbitrary units)
				Experiment 1			
ertrude	1	85.3	64.5	10.6	8.8	16.1	99
ontrol)	2	86.9	63.7	10.0	8.8	17.6	113
	3	94.8	62.1	9.2	8.5	20.3	116
oyce	1	92.4	62.2	10.0	9.0	18.7	167
hyroxine)	2	95.9	56.1	14.6	9.9	19.4	79
	3	94.5	60.9	9.3	9.0	20.7	129
unshine	1	88.2	63.4	7.6	8.6	20.4	121
hiouracil)	2	77.7	64.8	6.5	8.3	20.4	167
	3	82.4	63.3	7.9	8.0	20.8	132
				Experiment 2			
inky	1	86.7	63.9	8.9	7.2	20.1	112
ontrol)	2	86.0	65.9	8.5	7.2	19.0	121
	3	90.6	66.7	6.4	7.4	19.4	129
orothy	1	82.4	56.4	10.2	11.3	22.0	129
hyroxine)	2	92.9	51.5	17.5	12.1	20.7	43
	3	84.6	55.9	11.5	11.5	21.6	120
adfly	1	88.3	64.7	9.8	8.6	17.7	148
hyroxine)	2	94.5	57.3	14.7	10.3	18.2	64
	3	91.8	64.2	9.6	9.4	16.8	125
isty Morn	1	94.4	63.1	9.5	8.8	18.9	129
hiouracil)	2	85.1	67.8	6.1	8.1	18.2	213
	3	93.1	67.2	6.6	9.0	17.6	165
rixie	1	98.7	61.7	10.0	9.3	18.7	138
hiouracil)	2	92.0	66.6	5.5	7.7	19.8	209
	3	98.4	66.2	7.1	9.2	17.3	170

Table 8. Correlation of the content of ester-P and of inorganic-P in milk with the content of phosphatase

ow	Treatment in Period 2	No. of paired observations in all periods	Correlation between ester-P and phosphatase		Change of phosphatase per unit change of ester-P (i.e. linear regression coefficient)	Correlation coefficient of inorganic-P as % total P with phosphatase	Correlation of ester-P as % total P with phosphatase
			r	z			
ert-rude	None	30	-0.702***	-0.872	- 16.2	+ 0.402*	-0.683***
oyce	10 mg. Thyroxine/day	30	-0.947***	-1.801	- 17.0	+ 0.966***	-0.933***
un-hine	10 mg. Thiouracil/day	30	-0.978***	-2.240	- 24.3	+ 0.601***	-0.862***
inky	None	27	-0.764***	-1.005	- 10.0	+ 0.519**	-0.885***
oro-thy	10 mg. Thyroxine/day	27	-0.927***	-1.633	- 10.4	+ 0.827***	-0.896***
ad-fly	10 mg. Thyroxine/day	27	-0.960***	-1.946	- 9.5	+ 0.935***	-0.956***
isty orn	20 mg. Thiouracil/day	27	-0.953***	-1.862	- 18.3	+ 0.882***	-0.930***
rix-le	20 mg. Thiouracil/day	27	-0.866	-1.315	- 12.2	+ 0.818***	-0.845***

** P < 0.001
** P < 0.01
* P < 0.05

Table 9. Analysis of variance showing the curvilinearity of regression of ester P and phosphatase in the milk of the cow Gadfly

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Total	26	752.61	-	
Linear regression	1	693.55	693.55	293.63
Deviation from linear regression	25	59.06	2.362	
Deviation from curvilinear regression	24	25.49	1.062	31.61***
Curvilinearity	1	33.57	33.57	

Table 10. Correlation of the content of lipid-P in milk with the content of phosphatase

Cow	Period	Treatment in Period 2	Correlation coefficient	No. of paired observations	Change of phosphatase per unit change of lipid-P (i.e. linear regression coefficient)
			Experiment 1		
Gertrude	1) 2) 3)	None	+ 0.286	30	-
Joyce	1		+ 0.359	14	-
	2) 3)	Thyroxine	- 0.963***	16	- 69.5
Sunshine	1		+ 0.296	14	-
	2) 3)	Thiouracil	- 0.718**	16	- 30.5
			Experiment 2		
Dinky	1) 2) 3)	None	+ 0.047	27	-
Dorothy	1		+ 0.195	8	-
	2) 3)	Thyroxine	- 0.906***	19	- 34.3
Gadfly	1		+ 0.032	8	-
	2) 3)	Thyroxine	- 0.880***	19	- 38.1
Misty Morn	1		+ 0.064	8	-
	2) 3)	Thiouracil	- 0.745***	19	- 27.6
Trixie	1		- 0.049	8	-
	2) 3)	Thiouracil	- 0.805***	19	- 18.9

*** $P < .001$

** $P < .01$

Unstarred figures are not significant, i.e.: $P > 0.05$

Fig 6a

Relation between ester-P and phosphatase in the control cow Dinky.

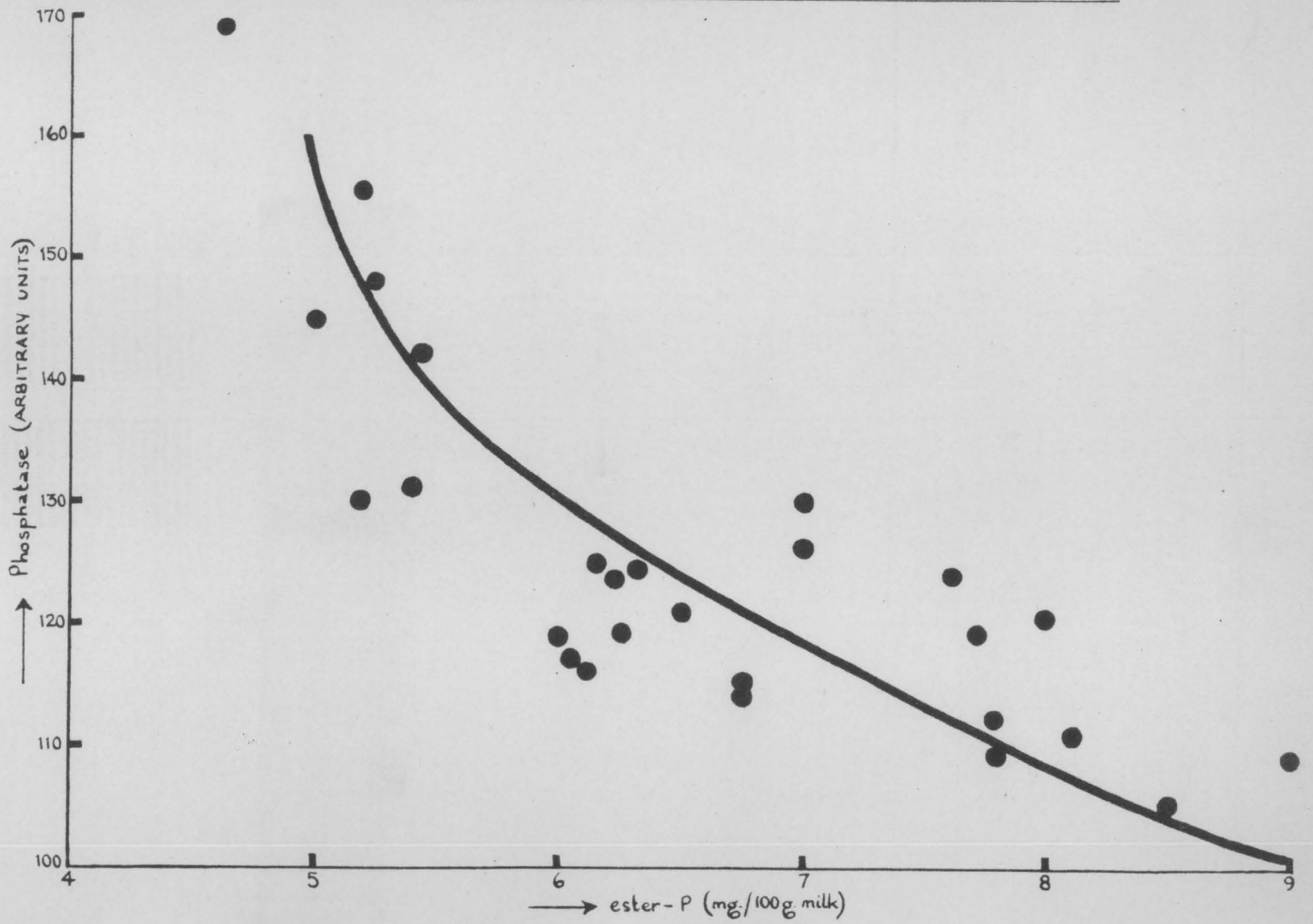


Fig. 6b. Relation between ester-P and phosphatase in the thyroxine Gadfly.

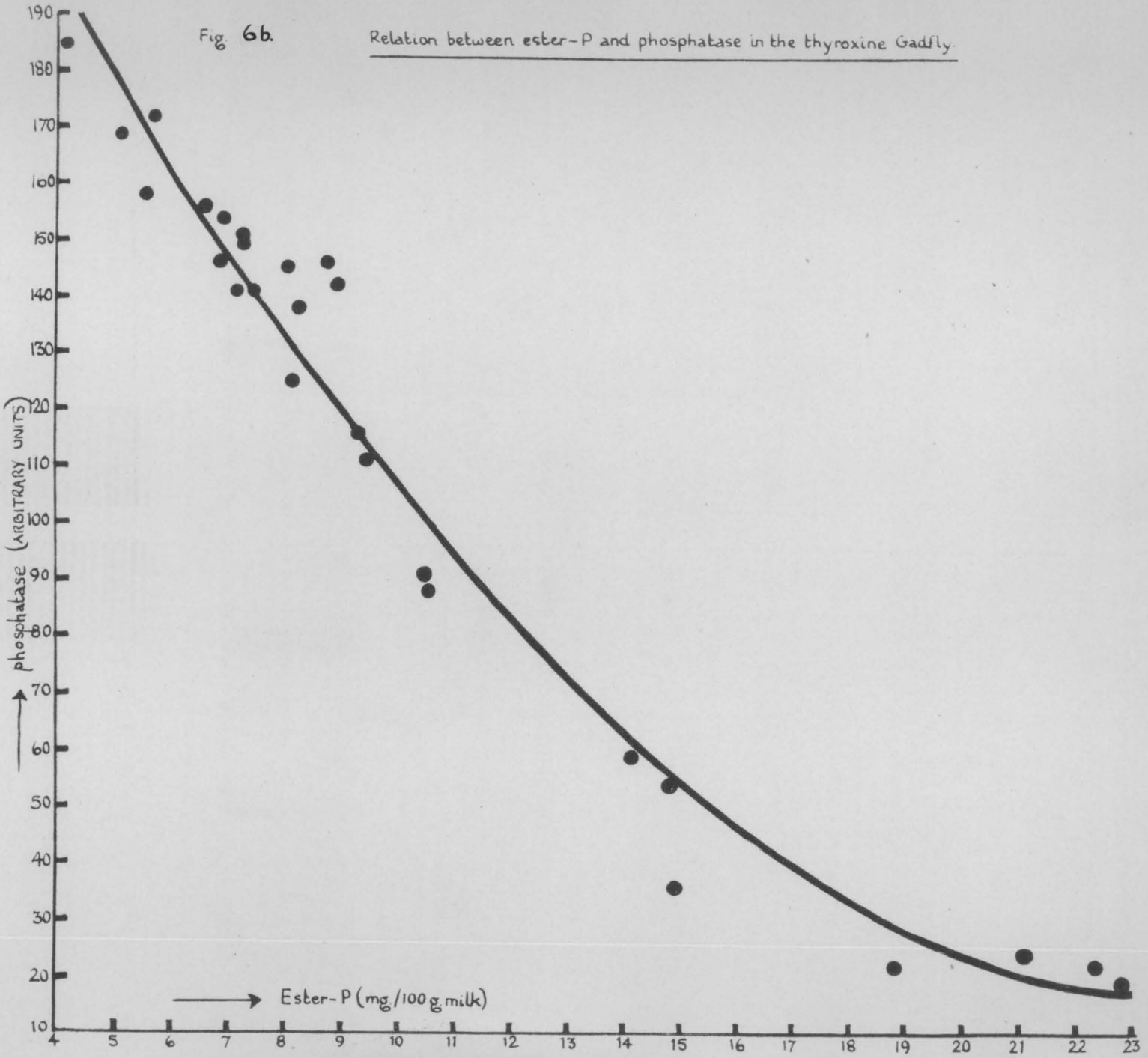


Fig. 6c. Relation between ester-P and phosphatase in the thiouracil cow Trixie.

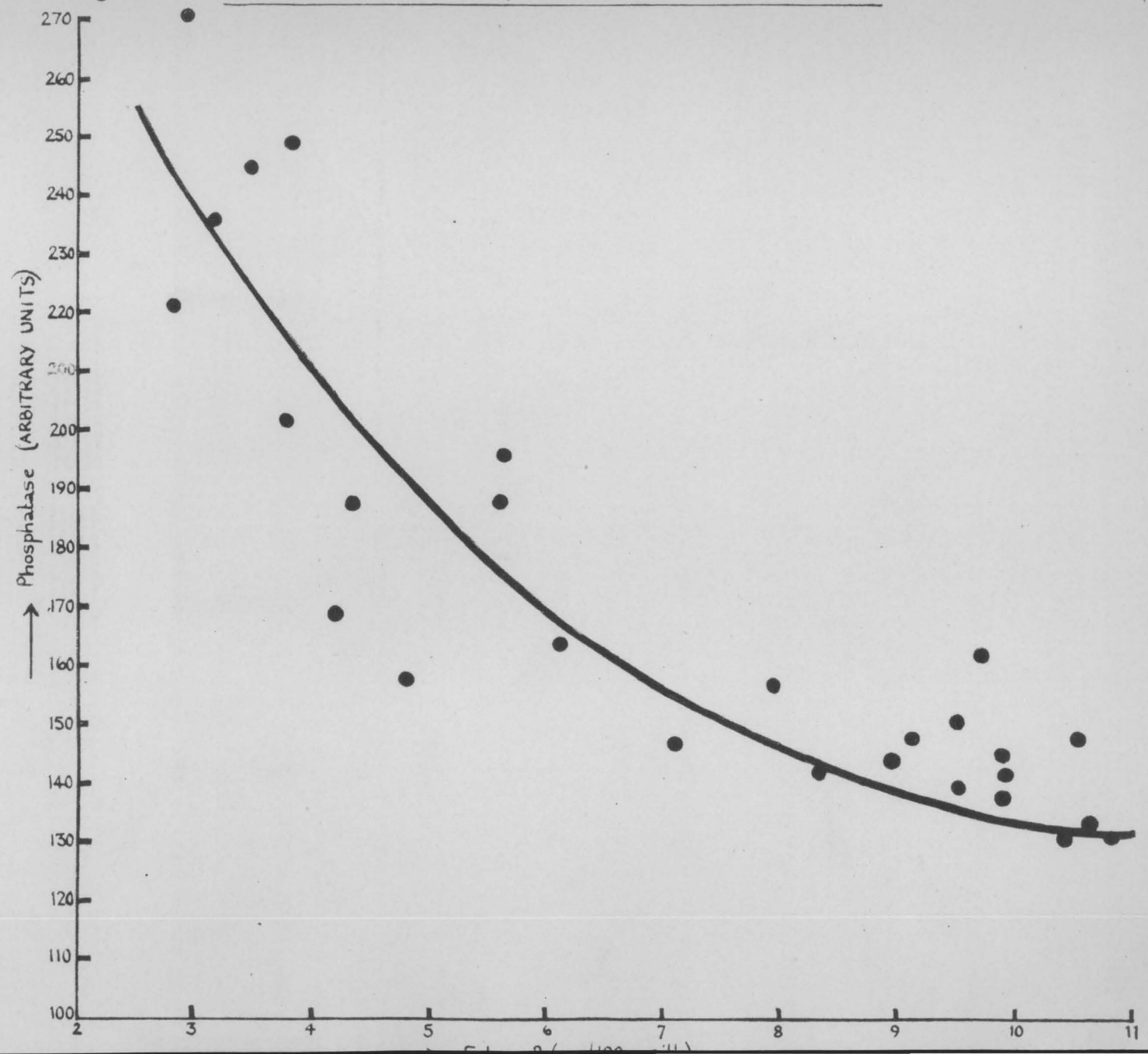


Fig. 7 Correlation between Inorganic P. (as % total P.) and Phosphatase

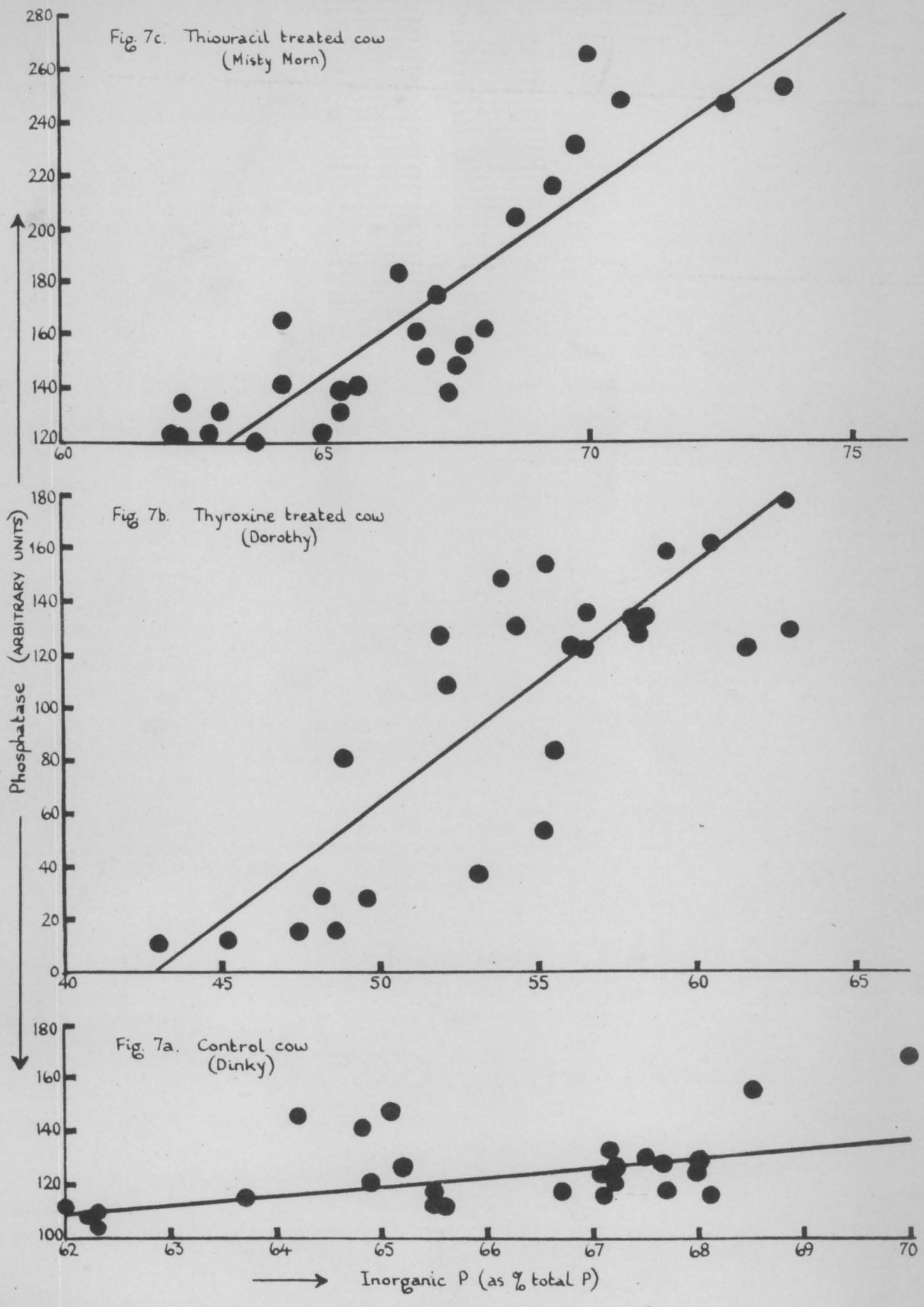


Fig. 8. Correlation between lipid-P and phosphatase

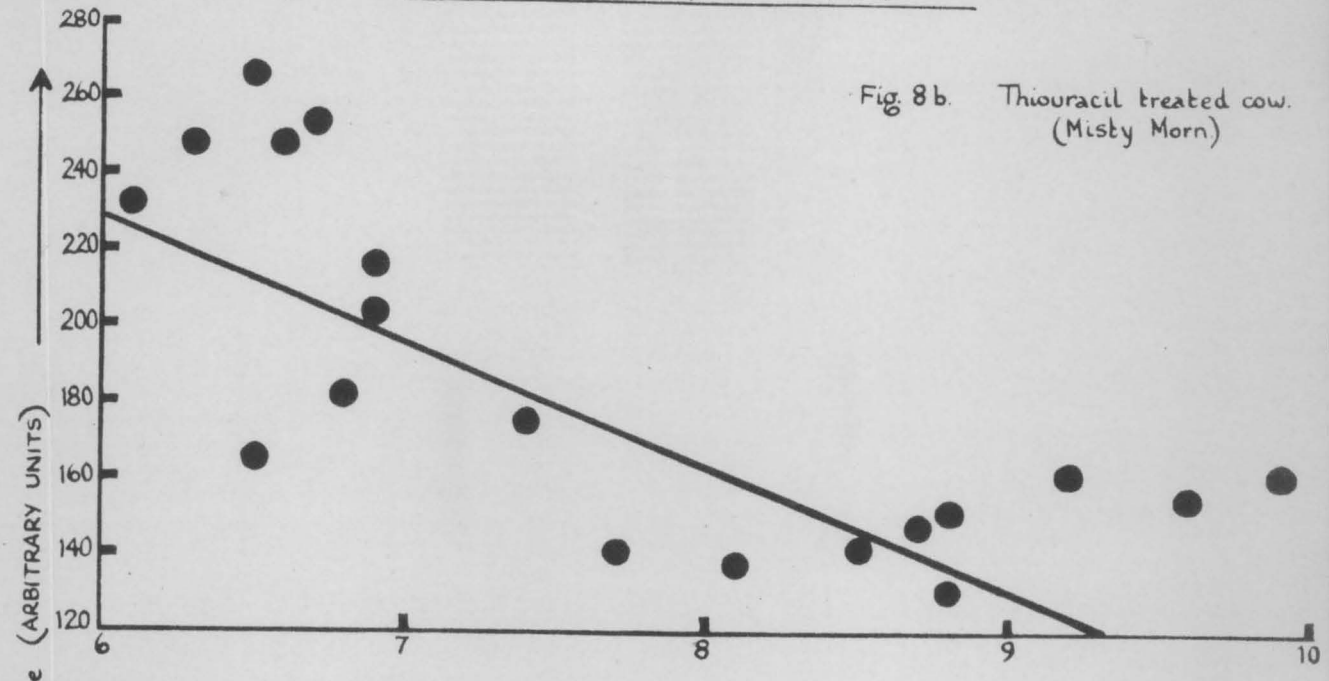


Fig. 8b. Thiouracil treated cow. (Misty Morn)

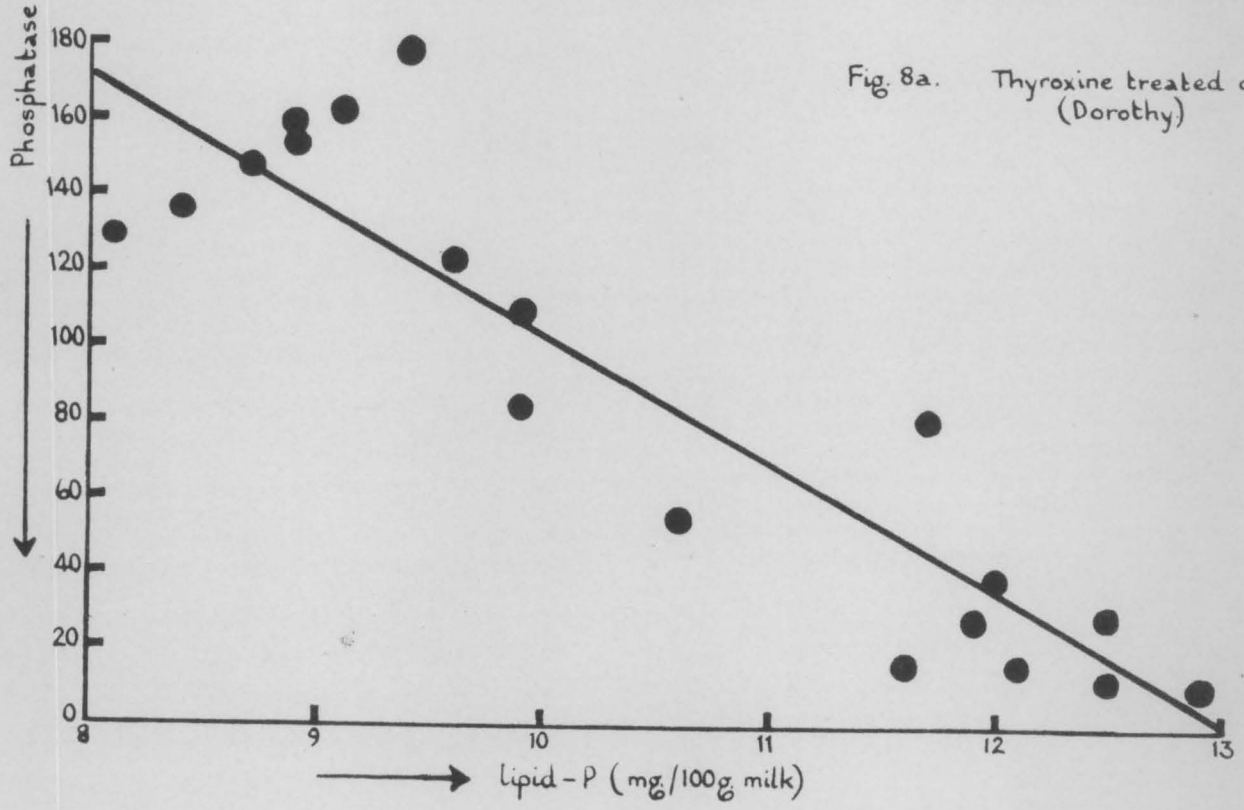


Fig. 8a. Thyroxine treated cow. (Dorothy)

Creatine and creatinine content of milk

It was decided to investigate the creatine contents of the milk to find whether the increase in ester-P caused by thyroxine may be explained partly by an increase in the labile phosphate such as creatine phosphate. The results of experiment 3 recorded in Table 11 show that neither thyroxine nor thiouracil caused any change in creatine or creatinine content of milk. An analysis of variance showed that there was no significant variation in creatine content of the milk between animals. There was, however, a significant variation between periods. This variation was attributable to advancing lactation since there was a regular trend of decrease during successive periods in all the cows. The lack of change in the creatine content of milk in thyroxine treated cows are in sharp contrast with that of urinary creatine which was markedly increased by thyroxine (Owen, 1948 a).

The statement of Basu & Mukherjee (1943) that ester-P in milk is exclusively the labile creatine phosphate is not supported by the present observations. When the results of creatine (Table 11) are considered in conjunction with the phosphorus partition (Table 7), it can be seen that in spite of large increases of ester-P caused by thyroxine, no changes occurred in the creatine content of milk. The amount of creatine present in milk can only account for 1.4 mg. ester-P, which is lower than the smallest ester-P figure observed

during thiouracil treatment. That the increase in ester-P in the milk of thyroxine cows was not due to an increase in creatine phosphate was further confirmed by allowing the trichloroacetic acid soluble fraction of the milk to stand with molybdate reagent which is known to catalyse the hydrolysis of creatine phosphate (Fiske & Subbarow, 1929). When the phosphate content of samples which had stood with molybdate reagent for 15 min. was compared with the phosphate content of samples which had stood for 1 hr. no difference was found.

Table 11. The creatine and creatinine content in milk of control cows and of cows receiving thyroxine or thiouracil

Cow and treatment in Period 2	Period	Total creatine as creatinine (mg./100ml.)	Creatine as Creatinine (mg./100ml.)	Creatine as % total	Creatinine (mg./100ml.)	Creatinine as % total
Sunshine (Control)	1	13.4	9.7	72.4	3.7	27.6
	2	12.8	9.2	68.7	3.5	31.3
	3	11.4	7.8	68.4	3.5	31.6
Ella (Control)	1	12.2	10.6	86.9	3.6	29.5
	2	14.0	9.9	70.7	4.1	29.3
	3	12.1	7.9	65.3	4.0	33.1
Gertrude (Thyroxine)	1	14.5	10.7	73.8	3.8	26.2
	2	13.4	9.4	70.2	4.2	29.8
	3	11.8	8.0	67.8	3.8	32.2
Dewdrop (Thyroxine)	1	12.9	9.6	74.4	3.3	25.6
	2	12.8	9.2	71.9	3.6	28.1
	3	11.1	7.8	70.3	3.3	29.7
Jassamine (Thiouracil)	1	13.3	9.6	72.2	3.7	27.8
	2	12.7	8.8	69.3	3.9	30.7
	3	10.7	7.1	66.4	3.6	33.6
Jenny (Thiouracil)	1	14.6	10.8	74.0	3.8	26.0
	2	13.8	9.7	70.3	4.1	29.7
	3	12.2	8.3	68.0	3.8	31.1

It was concluded therefore that the samples of milk collected from recorder vessels in the present experiments

in spite of being rich in ester-P, did not contain any creatine phosphate.

Total aneurin and the partition of aneurin

The results for total aneurin and the partition of aneurin in milk are illustrated graphically in Fig.9 for the two control cows (Figs.9a & 9b), the two thyroxine cows (Figs. 9c & 9d) and the two thiouracil cows (Figs.9e & 9f). In these diagrams the individual 2-day data throughout the experiment have been recorded. The numerical range of values is given in Table 12, and the average values for total aneurin in each period are recorded in Table 13, where the phosphatase and the percentages of aneurin present as free, cocarboxylase, and protein-bound forms are also recorded. As the cows in this experiment were in earlier stages of lactation than those used by Bartlett, Rowland & Thompson (1949), the values for total aneurin are higher than those reported by these workers. It can be seen from Fig.9 and Table 13 that the total aneurin gradually decreased in all the cows in successive periods. There were also large variations in the total aneurin values between cows. An analysis of variance of the total aneurin showed that the differences between cows and between periods are statistically significant. Due to the significant difference between cows comparisons cannot be made between the controls and the treated cows in the same period, and from a comparison of the same cow from period to period, the effect of the hormone treat-

ment is difficult to establish. As already mentioned there was a gradual decrease in total aneurin during the experiment, and it would appear that the rate of the decrease was slightly retarded by thyroxine and enhanced by thiouracil.

In spite of the failure of the hormones to affect any major changes in the total aneurin, the partition of the aneurin was considerably altered. Table 13 shows the large changes which occurred in the free aneurin fraction and the corresponding increase in the cocarboxylase fraction of the milk of the thyroxine animals. There was also a simultaneous decrease in the phosphatase and a small increase in the protein-bound aneurin. The full magnitude of these effects can be seen in Table 12 and in Figs. 9c and 9d. The decrease in the free form of aneurin in the milk of cows fed iodinated protein has been reported earlier by Bartlett et al. (1949). The present series of experiments has shown that this decrease is accompanied by an increase in the cocarboxylase and protein-bound aneurin when thyroxine is administered subcutaneously. At the peak of hormonal response, the cocarboxylase in the thyroxine cow, Dewdrop, accounted for 38% of the total aneurin compared with only 21% in the pre-treatment period. The present results showed further the effect of thiouracil in increasing the free aneurin and decreasing the cocarboxylase and proteinebound aneurin.

Fig. 9a

SUNSHINE

The Partition of Aneurin in Cow's Milk. (Expt. 3.)

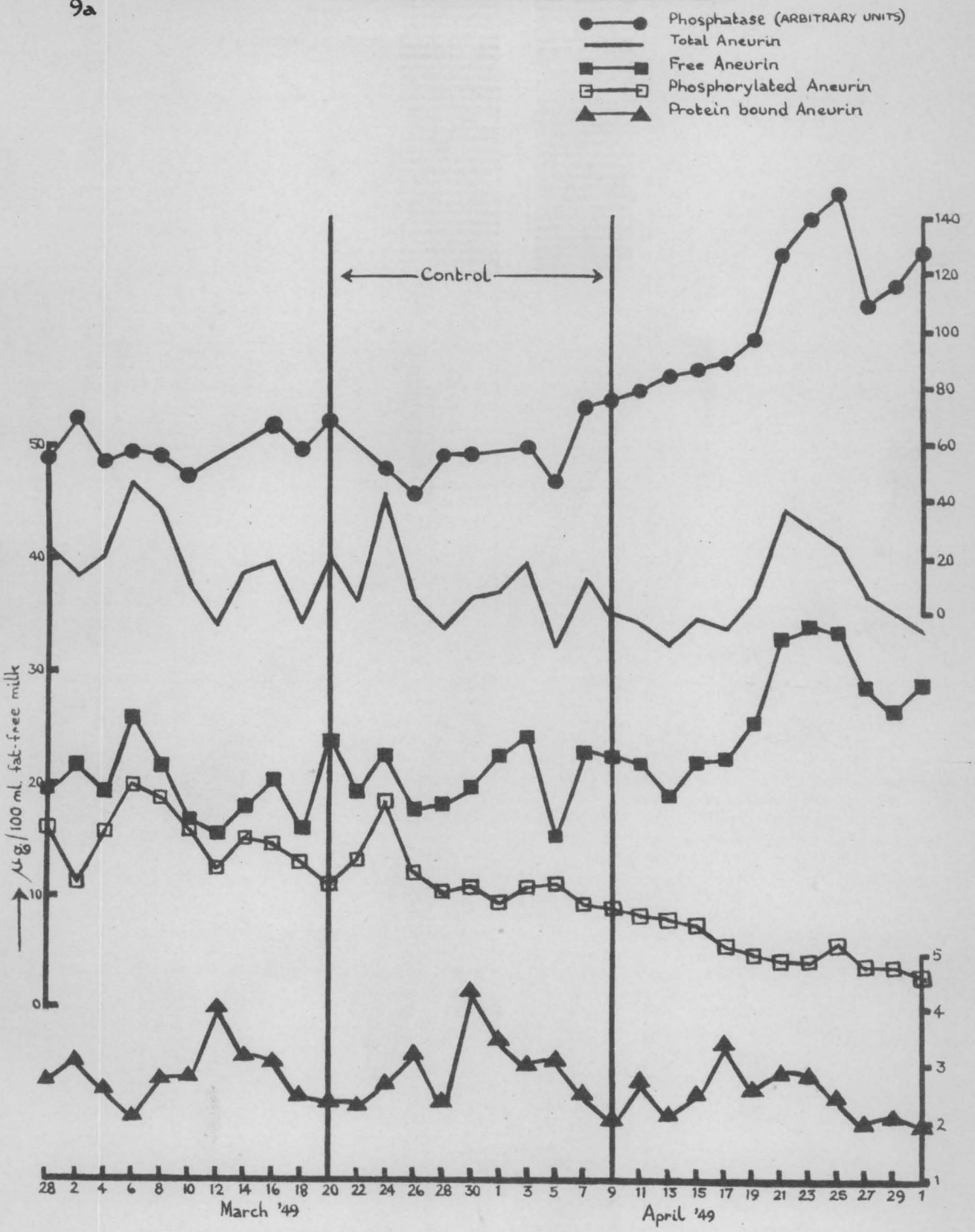


Fig. 9b.

ELLA

The Partition of Aneurin in Cow's Milk (Expt 3.)

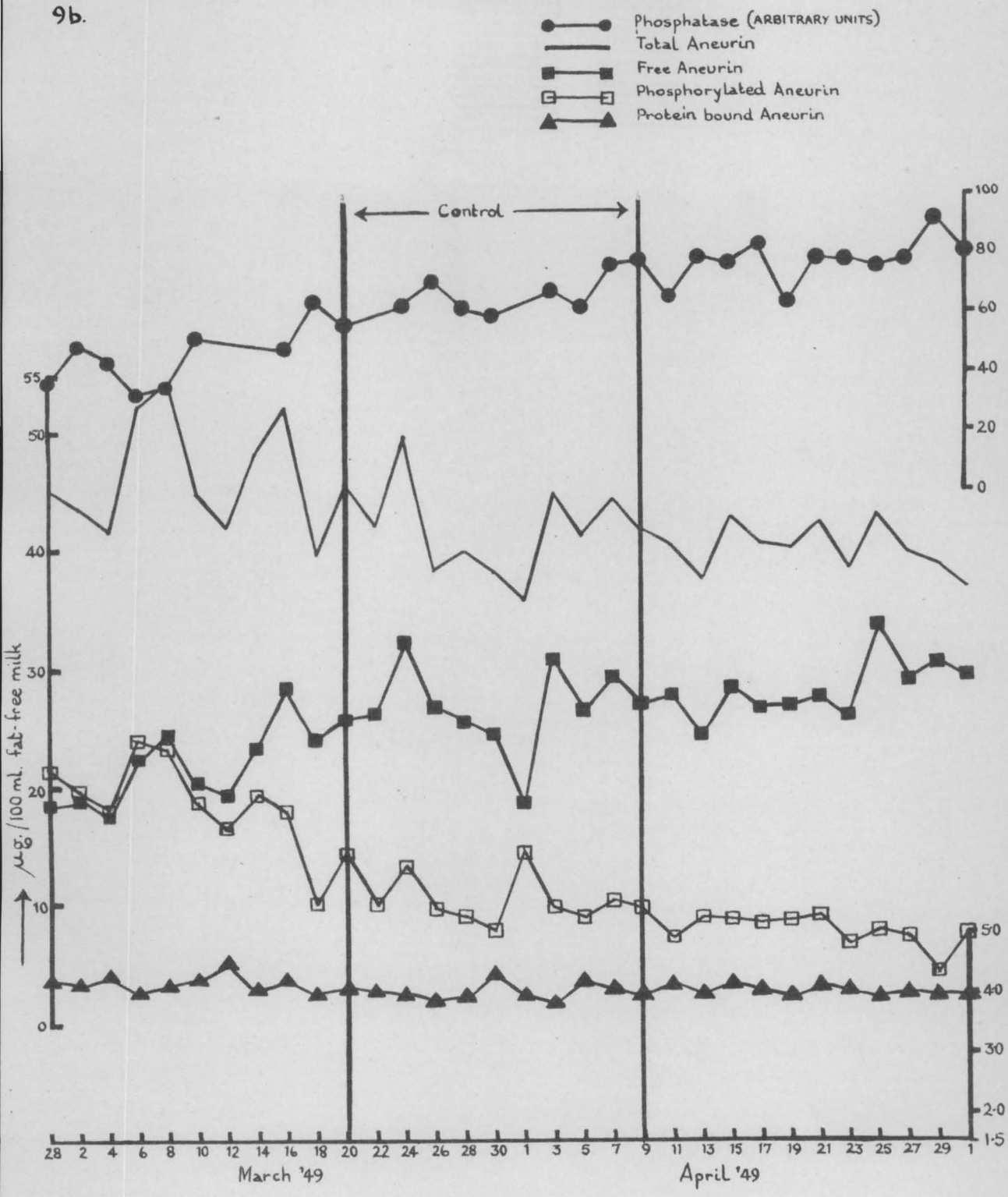


Fig. 9c. GERTRUDE

Effect of Thyroxine on the Partition of Aneurin in Cow's Milk (Expt 3)

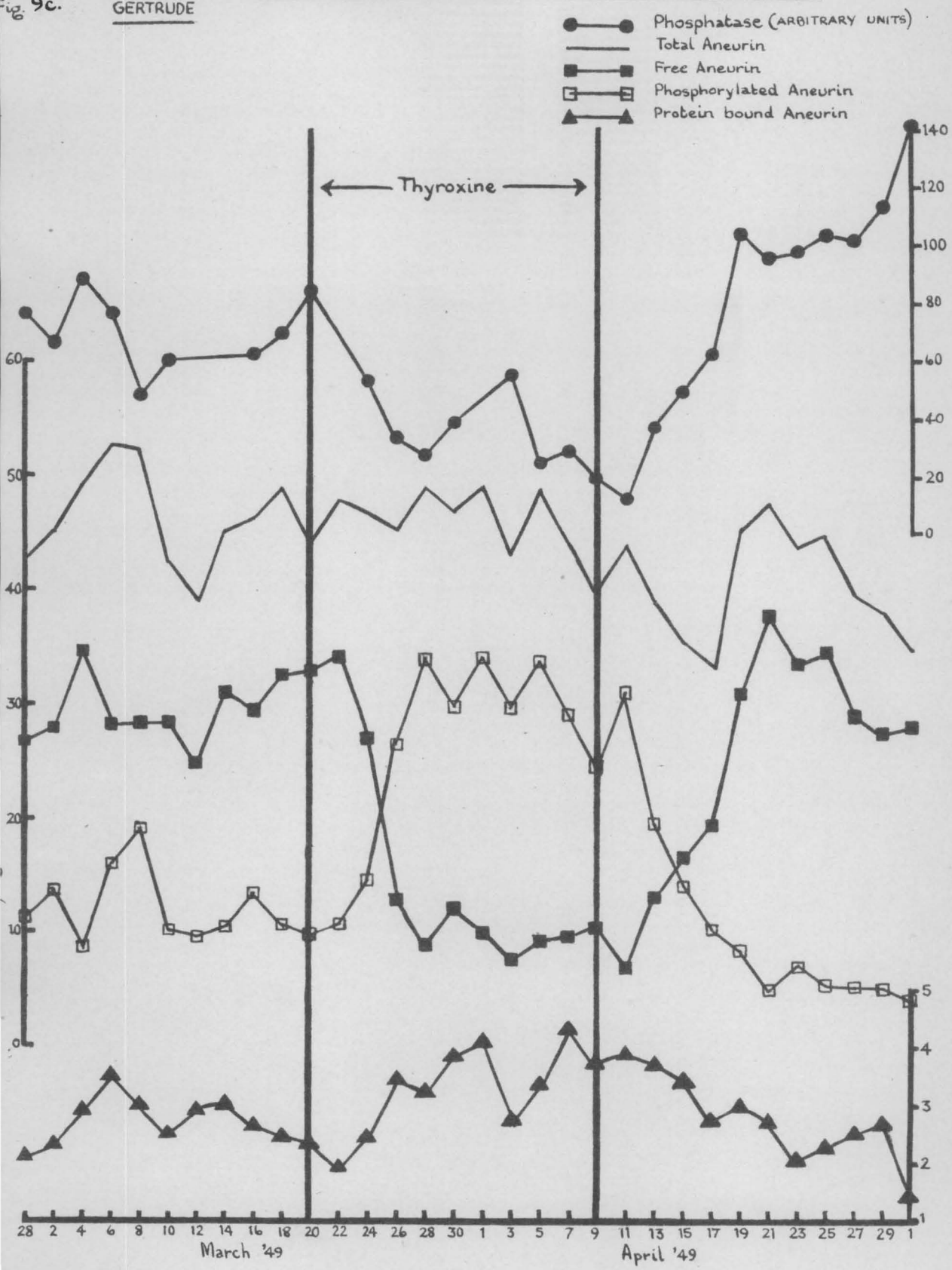


Fig. 9d DEWDROP Effect of Thyroxine on the Partition of Aneurin in Cow's Milk (Expt 3)

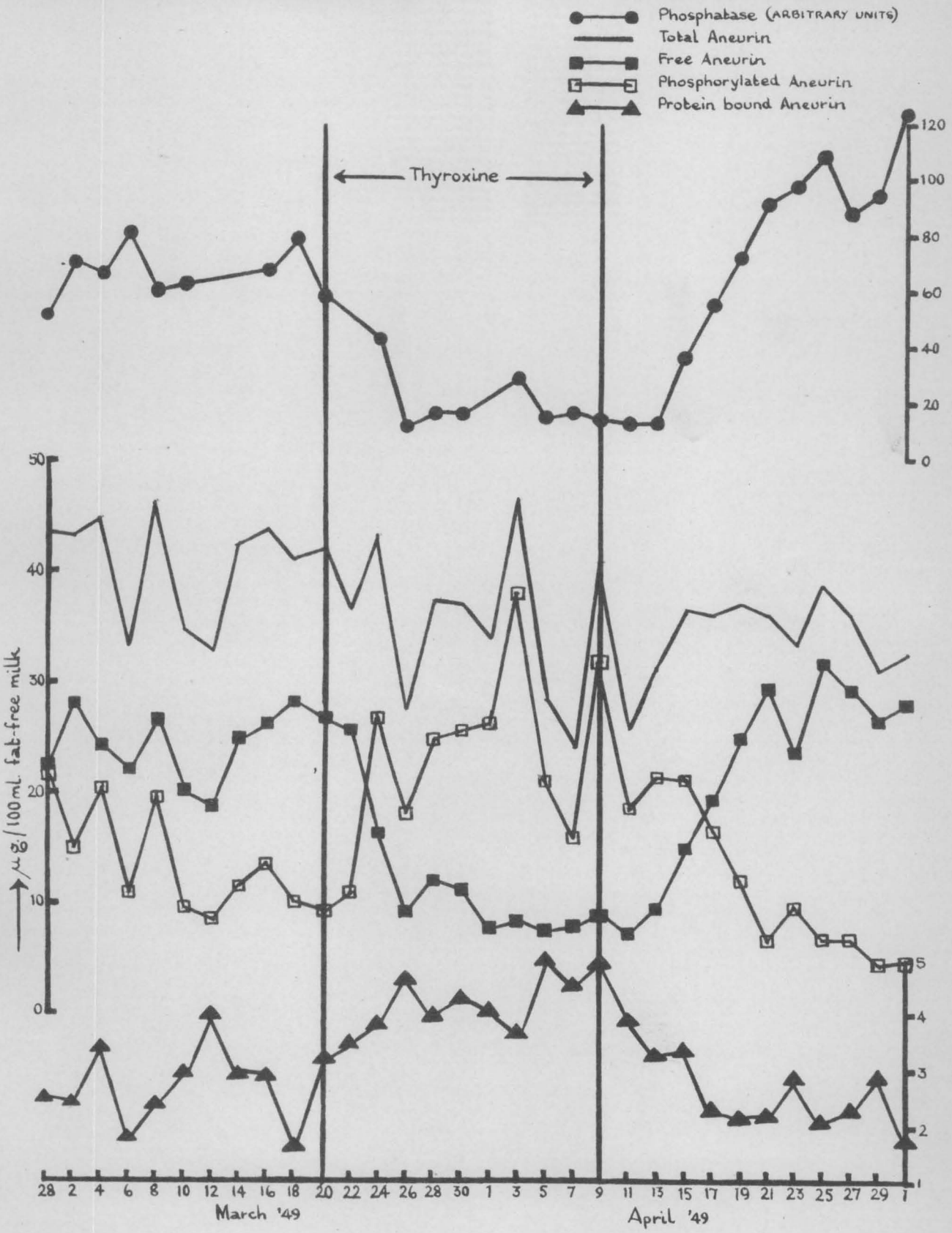


Fig. 9e JASSAMINE Effect of Thiouracil on the Partition of Aneurin in Cow's Milk (Expt. 3)

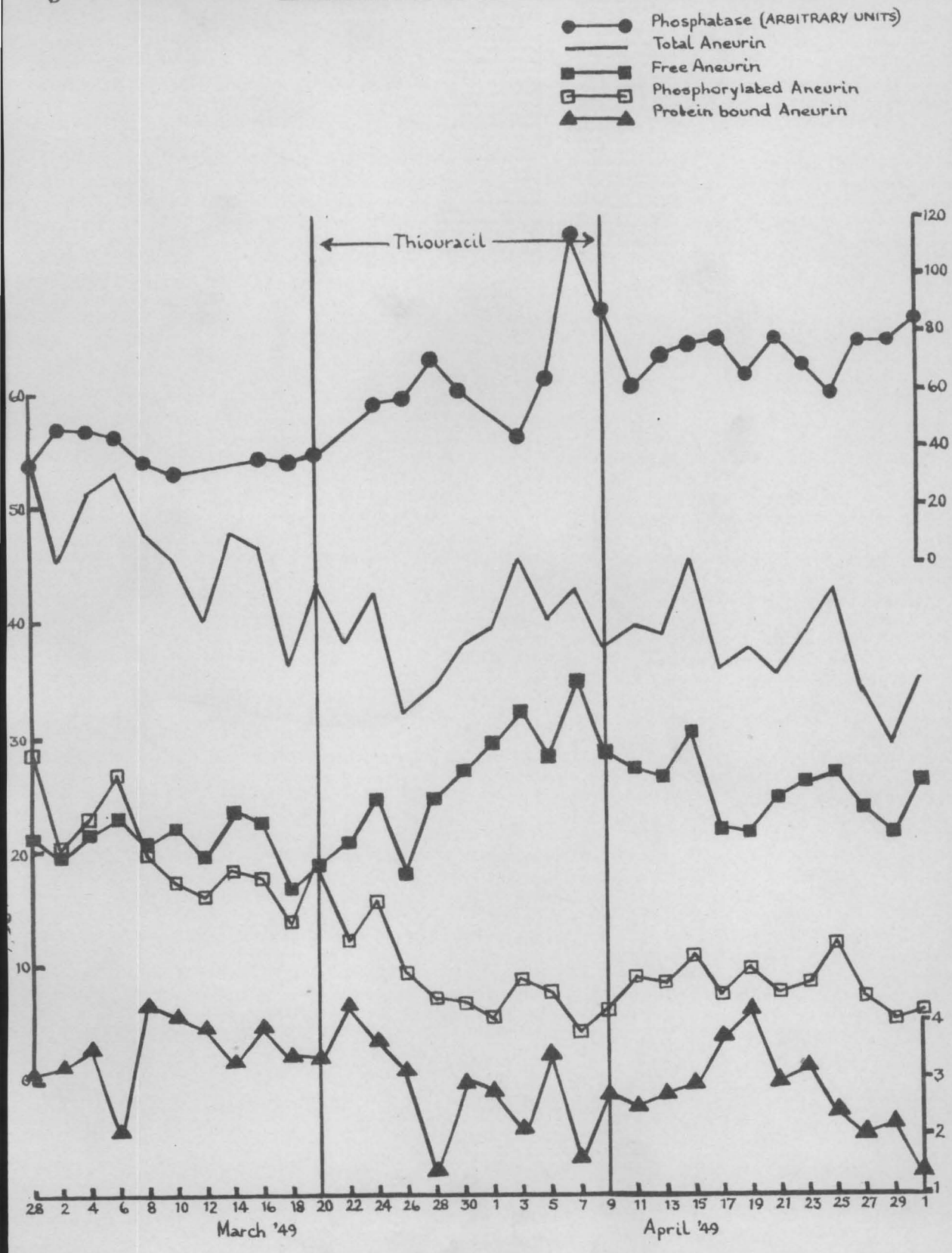


Fig. 9f. JENNY Effect of Thiouracil on the Partition of Aneurin in Cow's Milk. (Expt 3)

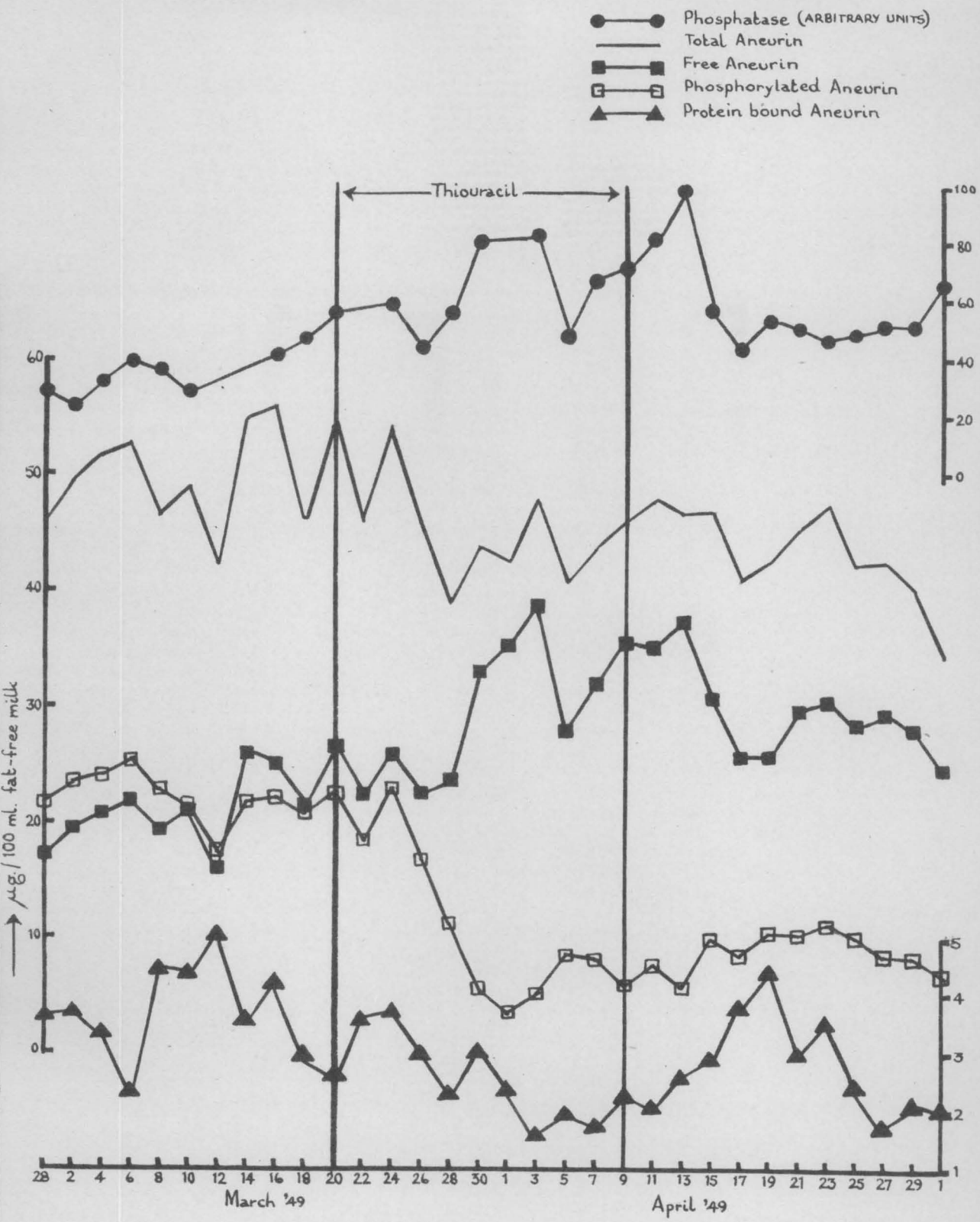


Table 12. The partition of aneurin in the milk of normal cows and cows receiving thyroxine or thiouracil

(Range of values)

Cow	Period	μg./100 ml. fat-free milk				Phosphatase (arbitrary units)
		Total aneurin	Free aneurin	Phosphorylated aneurin	Protein-bound aneurin	
Sunshine (Control)	1	34.2-46.6	15.8-25.7	11.2-20.0	2.1-4.0	48 - 69
	2	38.8-45.6	15.5-24.2	8.9-18.5	2.1-4.3	43 - 76
	3	32.4-43.5	19.2-33.0	3.0- 8.3	1.9-3.4	79 - 148
Ella (Control)	1	39.8-54.4	17.6-28.7	10.1-24.2	2.4-5.2	33 - 64
	2	41.3-49.8	18.6-32.2	7.9-14.4	1.9-4.1	59 - 78
	3	36.8-43.1	24.5-33.7	4.2- 9.1	2.1-3.3	66 - 92
Dewdrop (Thyroxine)	1	32.9-49.5	27.5-46.3	8.6-20.6	1.7-4.0	52 - 81
	2	30.4-49.9	24.2-46.5	11.1-38.0	3.5-5.0	12 - 43
	3	32.5-42.1	26.2-38.8	4.6-21.7	1.8-3.9	13 - 124
Gertrude (Thyroxine)	1	39.2-52.6	24.0-38.4	9.5-19.7	2.1-3.5	48 - 88
	2	39.7-48.9	7.7-34.1	10.9-34.0	1.9-4.3	19 - 55
	3	33.7-47.6	6.9-37.8	4.1-31.1	1.5-3.9	12 - 142
Jassamine (Thiouracil)	1	36.5-54.9	16.7-23.6	13.8-28.6	2.1-4.3	32 - 48
	2	32.2-45.3	18.0-35.0	4.1-15.6	1.4-4.3	56 - 114
	3	29.2-45.2	21.5-30.3	5.2-11.8	1.4-4.2	62 - 85
Jenny (Thiouracil)	1	42.2-55.9	16.0-26.1	17.6-25.3	2.3-5.0	24 - 56
	2	39.2-53.9	22.5-38.8	3.6-23.2	1.6-3.7	44 - 83
	3	34.4-47.7	24.8-37.3	5.7-11.1	1.7-4.4	44 - 98

Table 13. The partition of aneurin in the fat-free milk

Cow	Treatment in Period 2	Period	Total aneurin (µg./100ml.)	Percentage of total aneurin as Free	Cocarboxylase	Protein-bound	Phosphatase (arbitrary phenol units)
Gertrude	Thyroxine	1	46.5	64.2	26.2	5.9	70
		2	46.0	30.7	58.2	7.3	35
		3	41.1	62.2	26.2	6.9	84
Dewdrop	Thyroxine	1	42.0	58.1	31.5	6.7	67
		2	41.0	28.4	58.1	10.8	21
		3	39.2	56.9	30.9	7.0	73
Sunshine	None	1	39.4	50.4	38.0	7.3	59
		2	37.0	55.6	31.0	7.9	58
		3	37.1	72.7	14.9	6.9	109
Ella	None	1	46.5	48.0	39.9	7.6	47
		2	41.6	64.3	24.8	6.5	67
		3	40.5	70.6	18.5	6.6	78
Jassamine	Thiouracil	1	45.8	45.0	42.5	7.6	39
		2	39.1	68.4	21.1	7.3	70
		3	37.1	67.2	21.6	7.4	73
Jenny	Thiouracil	1	50.3	42.7	44.6	7.4	37
		2	45.0	66.8	23.4	5.7	64
		3	44.3	68.3	20.5	6.4	59

The same sort of effect was noticed in the control cows as in the thiouracil cows but it will be clear from Figs. 9e and 9f and from Table 12 that these effects were accelerated by thiouracil. Thus the minimum percentage of total aneurin in the form of cocarboxylase was decreased from 10% in period 1 to 8% in period 2 in the control cow, Ella, while the corresponding decrease was from 18% to 4% in the thiouracil cow, Jenny.

During the last period of the experiment, the combined effect of advancing lactation and the cessation of thyroxine treatment also produced some dramatic changes. The phosphatase titre was increased rapidly. There was a big drop in the cocarboxylase and a corresponding increase in the free aneurin. Protein-bound aneurin was also reduced. In the thiouracil cows the advancing lactation checked a sharp drop in the enzyme concentration but the opposite effects produced were noticeable during the first few days immediately following the cessation of treatment (Figs. 9e & 9f). The phosphatase tended to decrease and the protein-bound aneurin and cocarboxylase to increase. The full magnitude of the effect of advancing lactation can be seen in the control animal, Sunshine, in which phosphatase was markedly increased in period 3; the free aneurin was correspondingly increased and the phosphorylated forms were decreased. These effects were as dramatic as those produced artificially by thiouracil. This aspect of the effect of stage of lactation will

be referred to again later.

It can be seen from the graphs in Fig.9 that the changes in various forms of aneurin were related to the phosphatase content of the milk. A comparison of the phosphatase curve with those of the free and phosphorylated aneurin curves indicated the existence of definite correlations between these constituents. The coefficients of correlation between the enzyme and various aneurin fractions were therefore calculated, and it was found that there was a close positive correlation between phosphatase and free aneurin in the milk of all the cows. These correlations are recorded in Table 14. The correlation diagram is shown in Fig.10 for a control cow (Fig.10a), a thyroxine cow (Fig. 10b) and a thiouracil cow (Fig. 10c). It will be noticed from the table that the correlations held whether the results for free aneurin were expressed in units per 100 ml. milk or as percentages of the total aneurin, but that the highest correlations were obtained when the results were expressed on the latter basis.

As will be seen from Table 15 there was a highly negative correlation between cocarboxylase and phosphatase. The scatter diagrams are shown in Fig.11 for a control cow (Fig.11a), a thyroxine cow (Fig.11b) and a thiouracil cow (Fig.11c). The coefficients of correlation were all significant at 0.1% level.

Table 14. Correlation of the content of free aneurin in the milk with that of phosphatase

(No. of paired observations = 28)

Cow	Treatment in Period 2	Correlation ceofficient of free aneurin with phosphatase	Correlation coefficient of free aneurin as percentage of total aneurin with phosphatase
Gertrude	Thyroxine	+ 0.808***	+ 0.879***
Dewdrop	Thyroxine	+ 0.914***	+ 0.951***
Sunshine	None	+ 0.880***	+ 0.949***
Ella	None	+ 0.652***	+ 0.893***
Jassamine	Thiouracil	+ 0.594***	+ 0.842***
Jenny	Thiouracil	+ 0.878***	+ 0.833***

*** $P < 0.001$

Table 15. Correlation of content of cocarboxylase in the milk with that of phosphatase

(No. of paired observations = 28)

Cow	Treatment in Period 2	Correlation coefficient of cocarboxylase with phosphatase	Correlation coefficient of cocarboxylase expressed as percentage total aneurin with phosphatase
Gertrude	Thyroxine	- 0.926***	- 0.890***
Dewdrop	Thyroxine	- 0.780***	- 0.919***
Sunshine	None	- 0.849***	- 0.896***
Ella	None	- 0.901***	- 0.847***
Jassamine	Thiouracil	- 0.773***	- 0.826***
Jenny	Thiouracil	- 0.727***	- 0.787***

*** $P < 0.001$

Protein-bound aneurin was also negatively correlated with phosphatase. This correlation is shown in Fig.12. In this diagram the mean value of protein-bound aneurin for each period is plotted against phosphatase. An inspection of the plotted points and an analysis of variance of the regression (Table 16) indicated a significant departure from linearity. A curve was therefore fitted to the points by the method of least squares. The curvilinear regression shows that the changes in protein-bound aneurin were smaller where those for phosphatase were greater whether the changes in the concentration of the enzyme were natural or were induced by thiouracil.

Table 16. Analysis of variance showing significant departure from linearity of the regression of protein-bound aneurin on phosphatase

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Total	17	3.8058	-	-
Linear regression	1	2.8846	2.8846	-
Deviation from linear regression	16	0.9212	0.0576	-
Deviation from curvilinear regression	15	0.2236	0.0149	46.8***
Curvilinearity	1	0.6976	0.6976	

Fig. 10. Correlation between free-aneurin (% of total aneurin) and phosphatase

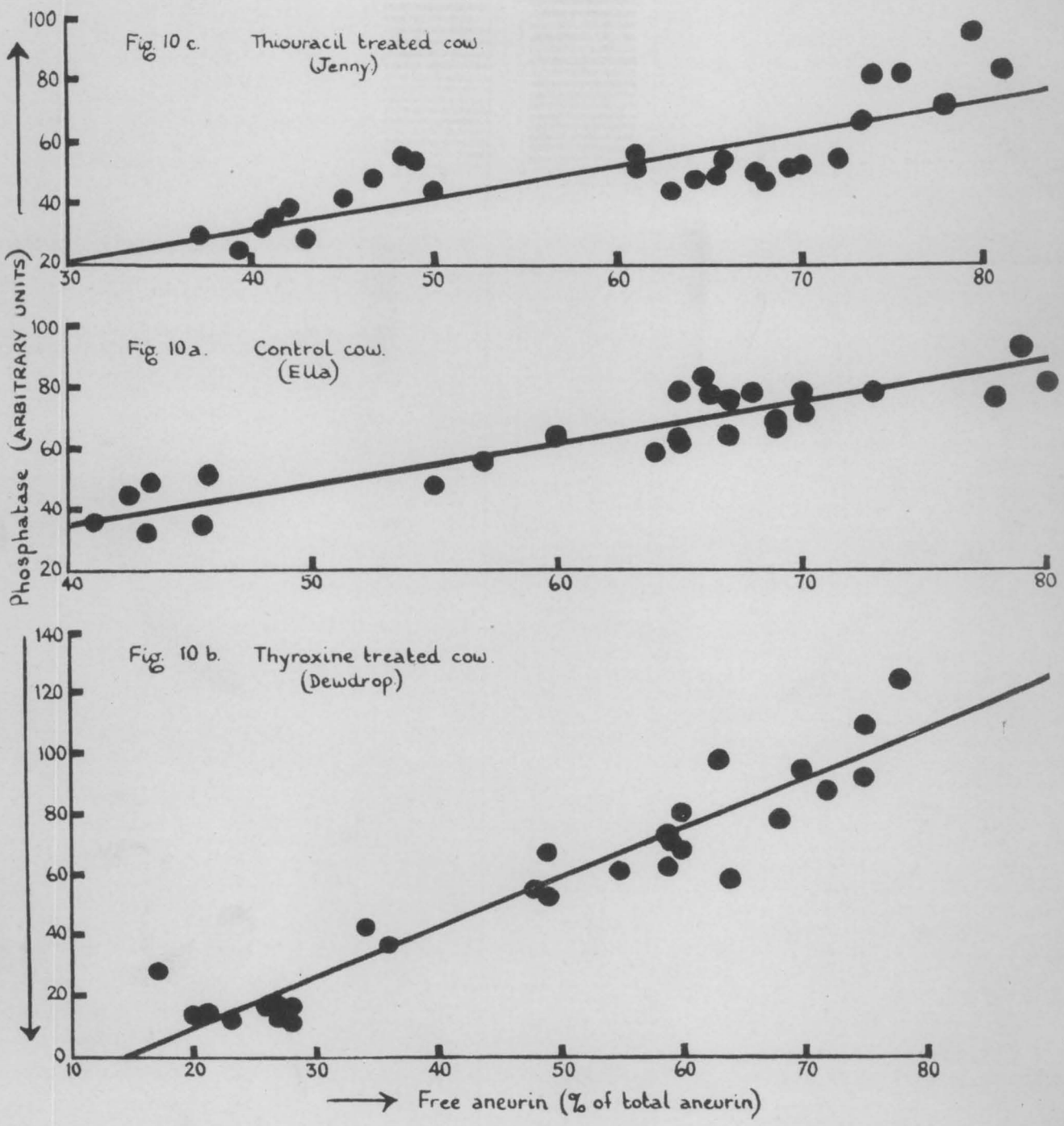


Fig 11a. Correlation between phosphorylated aneurin (cocarboxylase) and phosphatase.

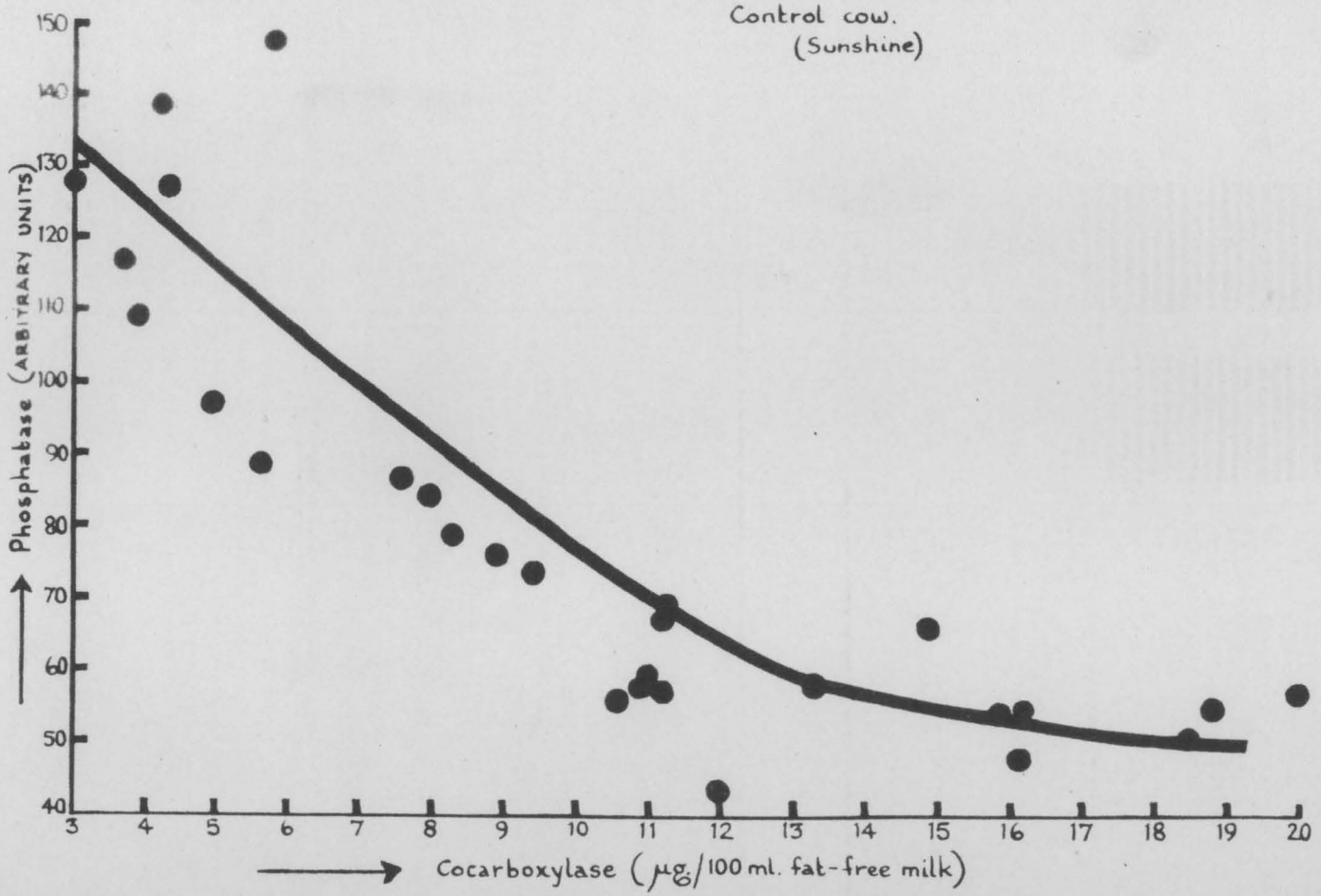


Fig. 11b

Correlation between cocarboxylase and phosphatase.

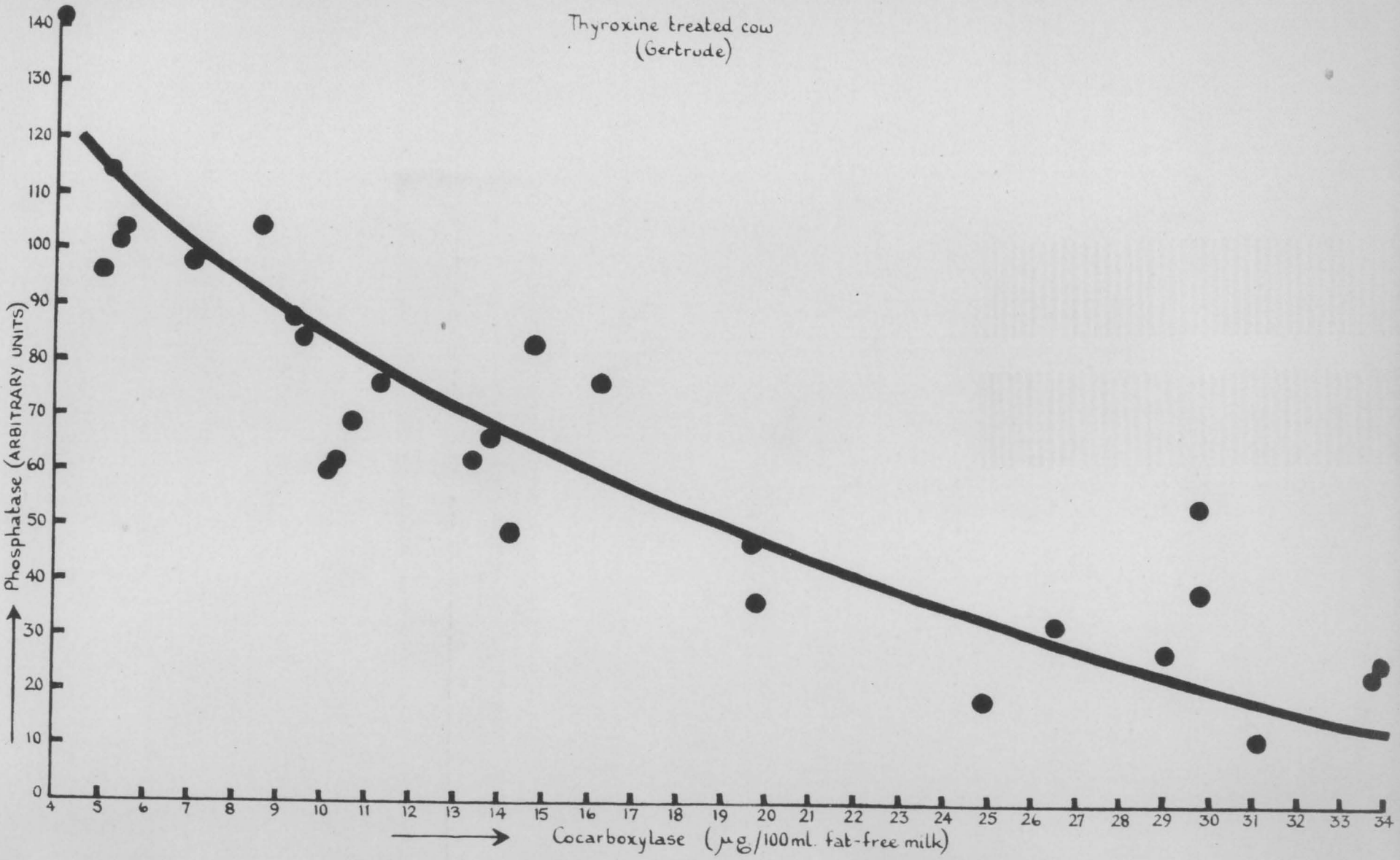


Fig. 11c. Correlation between cocarboxylase and phosphatase

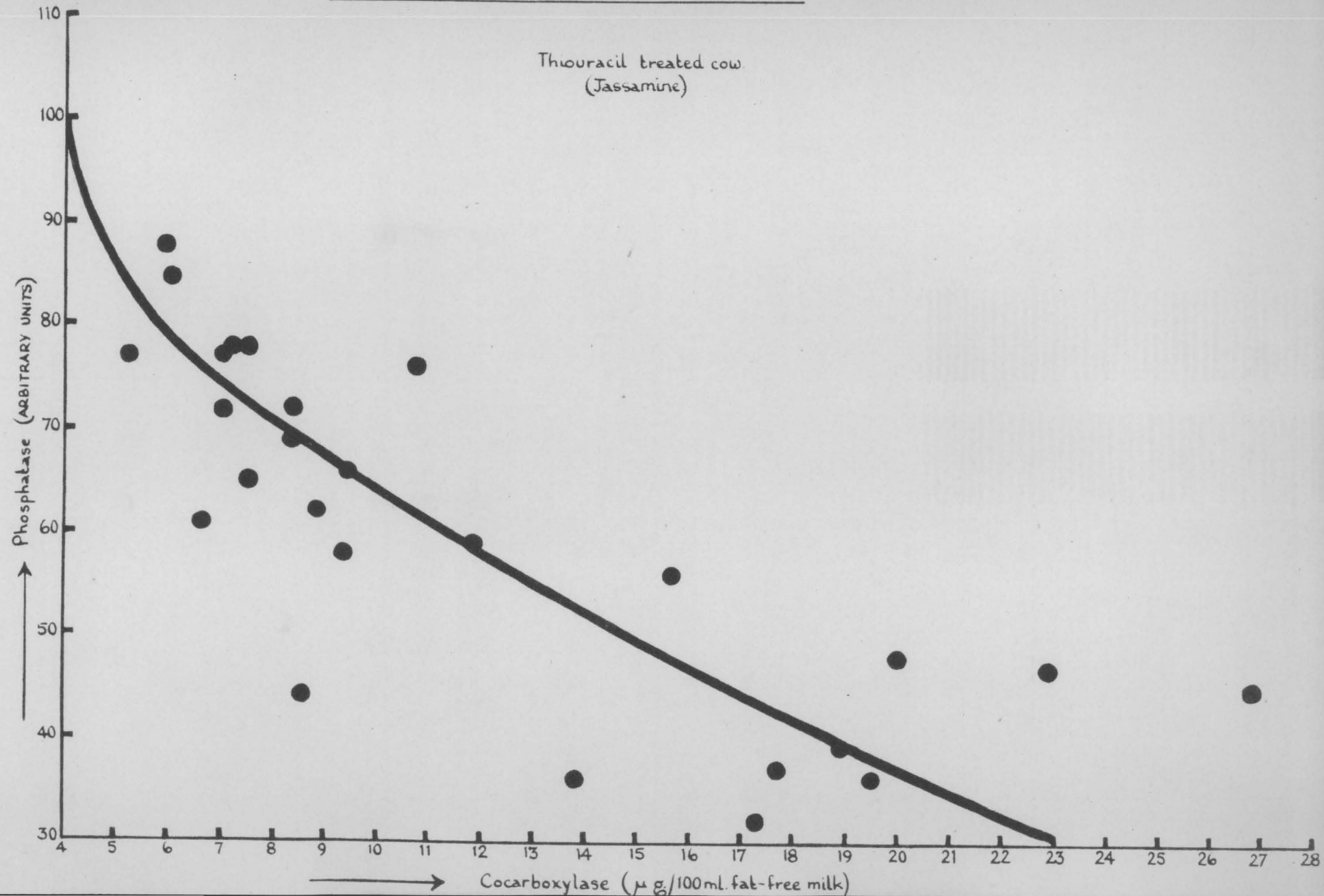
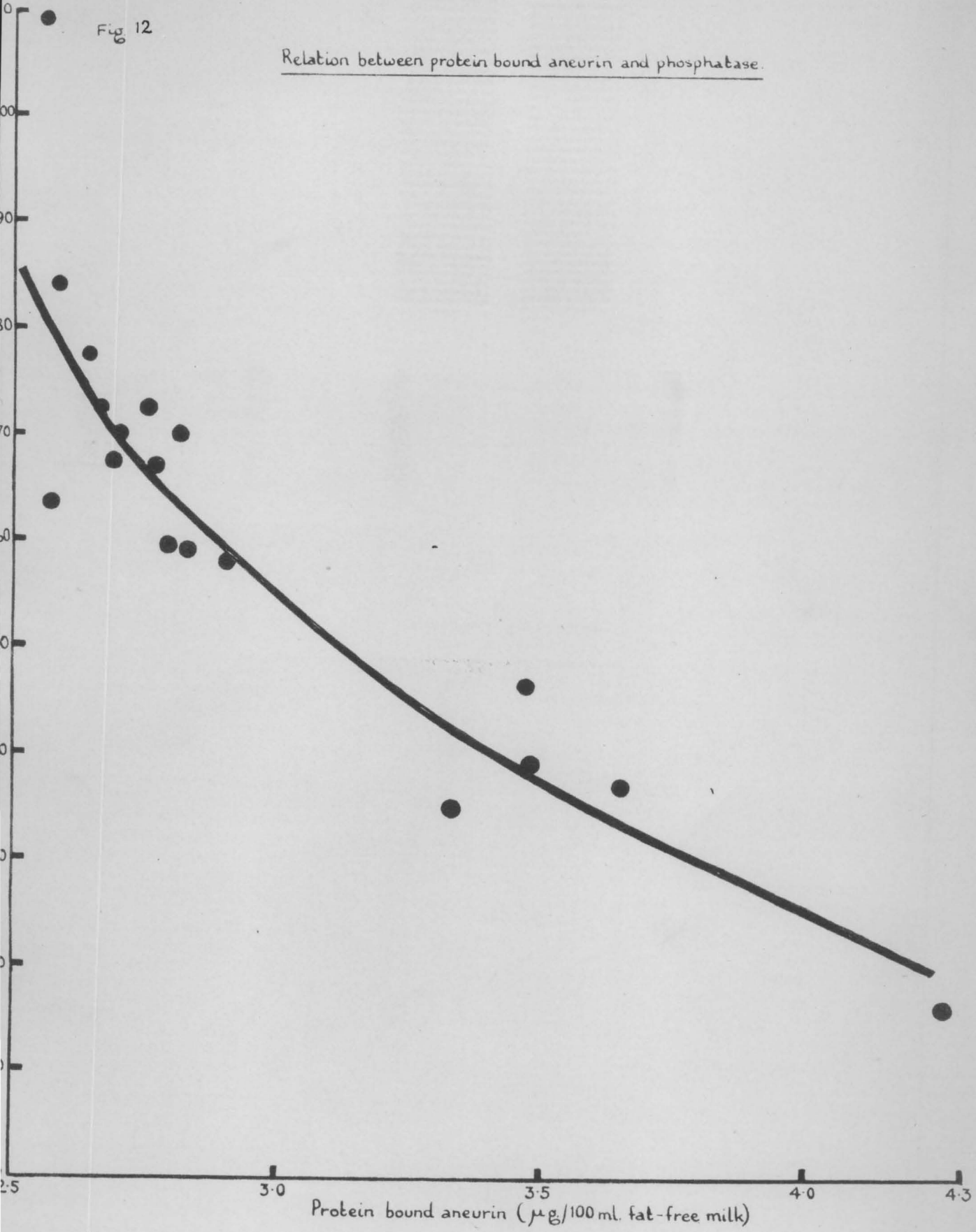


Fig. 12

Relation between protein bound aneurin and phosphatase.



Partition of phosphorus and aneurin throughout lactation, with special reference to colostrum

Before and after the experiments just described many milk samples were taken from the control cows and also from other cows of the Kirkhill herd in order to collect information on the effect of stage of lactation on the level of some of the constituents of the milk. Colostrum samples were also obtained from five cows which calved during the winter of 1948-49 for the determination of the phosphorus partition and from four cows which calved in the autumn of 1949 for a study of the aneurin partition. Total N was also determined in the first set of colostrum samples. The results for the N:P ratio in the milk and for the partition of phosphorus and aneurin are relevent to the discussion of the results already presented. These results are therefore briefly described below.

The results of N:P ratio in the first 14 days of lactation and in early, mid and late lactation are recorded in Table 17. They show that the N:P ratio was large in the first day's colostrum but dropped rapidly during the first week of lactation attaining a minimum value of 4.5 at the end of the first week when the yield of milk was approaching the maximum. Thereafter the ratio slowly increased and attained a maximum value of 7 in late lactation. There was only a small change in the ratio during early lactation. This low ratio during early lactation and the subsequent increase in late lactation is very consistent with similar

effects produced by thyroxine and thiouracil. Thyroxine appears to have a tendency to put the animal back to an earlier stage of lactation while thiouracil as it were tends to advance the stage of lactation.

Table 17. The changes which occur in the ratio of nitrogen to phosphorus in cows' milk as lactation advances

Days post partum	No. of cows	(mg./100g. milk) Total N	Total P	N:P	Milk yield (lb./day)
At parturition	3	2429	220	11.0	14.5*
1	3	1598	162	9.9	28.7
2	3	752	123	6.1	30.5
3	3	645	119	5.4	34.0
4	3	649	114	5.7	35.5
5	3	587	118	5.0	35.5
6	3	579	121	4.8	36.0
7	3	569	126	4.5	36.5
10	3	561	123	4.6	37.5
14	3	565	126	4.5	37.5
Early lactation (4th week)	6	563	120	4.7	38.5
Mid lactation (16th week)	3	585	96	6.1	28.0
Late lactation (40th week)	3	945	132	7.2	3.5

* only one milking

The results for phosphatase and the partition of phosphorus are recorded in Table 18. The phosphatase content in the first colostrum was high but it dropped to a minimum by the fifth day post partum. Thereafter it remained at a low level during early lactation but

increased rapidly during late lactation. The inorganic-P was high in the first colostrum but not as high as might have been expected from the high phosphatase figure. During the first few days post partum the inorganic-P decreased while that of ester-P was increased. Thereafter the changes were reversed, and became greater in mid and late lactation. With the large increase in phosphatase in late lactation, the inorganic-P was also increased while that of the ester-P was decreased. There was also a similar but less notable decrease in the casein-P as lactation advanced. The positive correlation between phosphatase and inorganic-P was highly significant. The correlation of ester-P with phosphatase was negative. These correlations obtained here under normal circumstances are similar to those produced artificially by hormonal treatment and reported earlier in the present chapter.

The partition of aneurin in colostrum and in milk from cows at different stages of lactation is recorded in Table 19. Cocarboxylase changed in the same way as ester-P with the progress of lactation while free aneurin followed the phosphatase. It will be noticed that in spite of the high aneurin figure obtained for the colostrum of the first few days, the level of free aneurin was low and was positively correlated with phosphatase. The changes in protein-bound aneurin were likewise negatively correlated with phosphatase. These changes are all highly consistent with those observed earlier in cows treated with thyroxine or thiouracil.

Table 18. Phosphorus partition in cows' milk as lactation advances

Days post partum	No. of cows	Phosphorus (mg./100 g. milk)					Phosphatase (arbitrary units)
		Total	Inorganic	Ester	Lipid	Casein	
At parturition	5	232.4	136.7	25.4	26.2	44.1	167
1	5	178.5	108.3	20.3	16.4	33.5	98
2	5	127.6	65.1	17.4	18.5	26.6	35
3	5	121.4	65.7	18.1	17.3	20.3	19
4	5	123.5	67.4	18.4	17.5	20.2	16
5	5	120.1	62.2	19.2	17.3	21.4	11
6	5	124.0	61.2	20.5	19.1	23.2	14
7	5	129.5	68.3	18.2	20.5	22.5	17
10	5	131.2	66.5	20.5	19.5	24.7	12
14	5	135.3	69.5	21.6	19.6	24.6	10
Early lactation (4th week)	4	116.4	62.9	16.3	16.5	20.7	37
Mid-lactation (16th week)	3	95.6	60.5	7.4	10.2	17.5	125
Late lactation (40th week)	3	125.3	90.4	5.3	12.4	17.2	195

Table 19. Partition of aneurin in cows' milk as lactation advances

Days post partum	No. of cows	Aneurin (g./100 ml. fat-free milk)				Phosphatase (arbitrary units)
		Total	Free	Cocarboxylase	Protein-bound	
At parturition	4	74.3	38.2	20.3	14.4	154
1	4	65.6	27.4	28.6	8.2	88
2	4	60.8	22.1	32.5	5.2	32
3	4	62.3	16.6	37.6	6.7	17
4	4	57.4	14.9	34.1	7.3	12
5	4	52.1	13.8	30.4	7.6	10
6	4	53.5	14.1	30.7	7.7	11
7	4	52.6	14.4	29.7	7.1	14
10	4	47.6	13.7	24.2	7.4	16
14	4	48.2	13.9	25.6	7.5	12
Early lactation (5th week)	6	43.6	24.2	14.4	4.1	54
Mid-lactation (16th week)	4	33.5	23.9	5.7	3.2	138
Late lactation (38th week)	3	27.8	23.2	2.3	1.7	189

Discussion

The ratio of N:P in the milk shows variations attributable to the thyroid gland. Thus in the present work thyroxine increased while thiouracil decreased the phosphorus content of milk. Nitrogen content (Figs. 4b & 4c) was not affected by either drug. As shown in Table 5, therefore, the ratio of N:P was decreased by thyroxine and increased by thiouracil. Normally the N:P is lowest at the peak of lactation and is increased in late lactation (Table 17). The ratio is thus inversely related to the activity of the mammary gland. There is also reason to believe that this ratio is characteristic of the species from which the milk comes. In cow's milk this ratio is only 6:1 while in human milk it is 20:1 (c.f. Chapter IV). Even after treatment of the cows with thiouracil the N:P ratio in cow's milk is still much smaller than that of human milk.

It has been shown that during thyroxine or thiouracil treatment the phosphorus content of the casein fraction is very variable tending to increase with thyroxine and decrease with thiouracil (Fig. 5). This phenomenon also occurred in normal lactation, the casein fraction becoming poorer in phosphorus in late lactation (Table 20). This difference of composition may be correlated with the well-known difficulty of preparing casein of constant phosphorus content (Associates of Rogers, 1935).

The dramatic changes in milk phosphatase (Figs. 1 & 2) caused by treatment with thyroxine or thiouracil are of considerable importance in understanding the biochemical factors which govern milk secretion and control milk composition. The present results (Table 8 & Fig.6) show further that, in milk, the enzyme phosphatase is inversely correlated with its substrate. What part this phenomenon plays in mammary secretion is a matter for conjecture. It is noteworthy, however, that as ester-phosphorus increases, both lipid and casein phosphorus increase with it (Fig.5) and these increases are partly at the expense of inorganic phosphorus (Tables 7 & 8). These observations are consistent with current theories of biochemical phosphorylation in which it is supposed that inorganic phosphate passes by way of ester-phosphorus into the more complex lipid and casein phosphorus. Zilversmit, Entenmann & Chaikoff (1948) found that the ester-phosphorus compound, glycerophosphate, is the immediate precursor of phospholipins. More recently Popják & Muir (1950) found further that the reaction between glycerophosphate and phospholipins is reversible. In the present experiments it was found that an increase in ester phosphorus was associated with an increase in lipid phosphorus when thyroxine was given (Fig.5c). Similarly a decrease in ester phosphorus in cows treated with thiouracil was associated with a decrease in lipid phosphorus (Fig.5f).

When the gland is made to work faster, as it is by thyroxine, more of the intermediate substance, ester

phosphorus, is elaborated and less of the raw material, inorganic phosphate, is left. But if as shown in experiments with livers of rats (Williams-Ashman, 1948) thyroxine increases the concentration of enzymes in the mammary epithelium, it is difficult to explain why, when thyroxine is given, the amount of phosphatase in milk becomes less. Perhaps the explanation of the lessening output of phosphatase in milk at a time when the blood contains more (Folley & White, 1936) is to be related to the power of cow's body, when treated with thyroxine, to retain phosphorus (Owen, 1948b) even while losing calcium and retaining nitrogen less avidly. When the body works faster as it does under the influence of thyroxine, this avid retention of phosphorus is probably related to an increase in the concentration of enzymes in the body and with an increased synthesis of the energy-transferring phosphoric esters which are indispensable to their action. Under such conditions it is not surprising that the phosphorus supplies of the soft tissues are maintained partly at the expense of the bones. Phosphorus passes from the bones via the soft tissues to the milk and the equivalent of calcium passing with it from the bones, being surplus to the low requirements of calcium by soft tissues, is passed out of the body in the faeces of the cow (Owen, 1948b). That thyroxine induces large losses of calcium from the body is well known. Hunter (1930) showed this in man, Parhon & Derevici (1932) in the dog, Blaxter (1948) in the sheep and Owen (1948b) in the lactating cow.

From the higher phosphatase content observed in the blood (Folley & White, 1936) when there was less in the milk (Figs. 1 & 2) it is tempting to assume that the function of the phosphatase in the gland is synthetic even though in milk as secreted its activity is catabolic, for it hydrolyses ester-phosphorus present in milk (Graham & Kay, 1934). The reversibility of phosphorylases is well attested. When, under the influence of thyroxine, the activity of the mammary gland is accelerated (Fig.4), more of the enzyme is perhaps utilised in synthesising ester-phosphorus and lipid phosphorus and consequently less passes into the milk. At the same time the synthetic products, ester-P and lipid-P, find their way into the milk in larger amounts (Fig. 5c). This hypothesis also explains the reverse changes (Fig.5f) in the thiouracil cows and further weight is lent to it by the negative correlations between phosphatase on the one hand and ester-P and lipid-P on the other.

The increase in the synthesis of various phosphorylated esters during thyroxine treatment would also necessitate an increase in phosphorylated co-enzymes which are so important in the body's energy transfers. The results show that like ester-P, phosphorylated aneurin and protein-bound aneurin in milk were increased by thyroxine and decreased by thiouracil (Table 13). Normally a higher proportion of phosphorylated aneurin is a characteristic of early lactation (Table 19). In late lactation the amount of phosphorylated aneurin is about 10% of the total aneurin or in other words the

ratio of total to free aneurin approaches unity. The larger amount of phosphorylated aneurin after thyroxine appeared to occur at the expense of free aneurin because the total aneurin remained constant (Table 13). With thiouracil the proportion of free aneurin was increased apparently at the expense of phosphorylated fractions.

The negative correlations between phosphorylated compounds and phosphatase have thrown some light on the biochemical aspect of milk synthesis by the mammary gland. Nevertheless it is realized that concomitant variations can only provide circumstantial evidence, and give no indication which of two correlated phenomena is the cause and which is the effect.

General conclusion

The composition of milk from cows treated with thyroxine are comparable with those of early lactation milk. Both these conditions are indicative of higher activity of the mammary gland which is reflected in a lower N:P ratio, a lower phosphatase, a lower inorganic-P and a higher content of phosphorylated organic compounds. The composition of milk with respect to minor constituents from thiouracil cows, resembles that of the milk of cows approaching the end of lactation. In all these cases the N:P ratio and phosphatase are larger and the content of phosphorylated compounds is smaller. The changes on these minor constituents are more clear cut than any changes in the major constituents. The many synthetic activities of the body are higher at early lactation and during thyroxine treatment. In

contrast synthetic activities are impaired in late lactation and during thiouracil treatment.

Close relationships between phosphatase and various phosphorylated compounds have been observed throughout this study and were found to hold whether the animals were at different stages of lactation or were treated with drugs. It is justifiable to conclude from the information available that in milk a low phosphatase concentration is always associated with a high concentration of phosphoric esters and conversely, irrespective of the root cause of change of phosphatase.

Summary

A study has been made of the effects of thyroxine and thiouracil on the concentration of some of the constituents of cows' milk. Data have also been obtained on the effects of the stage of lactation on the composition of the milk. The main results can be summarised as follows :-

(1) Thyroxine decreased the phosphatase titre of milk and thiouracil increased it. The titre was found to be low during early lactation but to increase considerably in late lactation.

(2) Comparison of the rateof the increase of the phosphatase titre after hormonal treatment, showed that 20 mg. thiouracil increased the phosphatase level as much as 10 mg. thyroxine decreased it.

(3) Neither thyroxine nor thiouracil affected the partition of nitrogen in the milk, between casein, albumin, globulin, proteose-peptone and non-protein nitrogen. Total nitrogen also remained practically unchanged.

(4) Thyroxine increased and thiouracil decreased the content of phosphorus in the milk. Thyroxine decreased and thiouracil increased the ratio of total nitrogen to total phosphorus in the milk. Analysis of colostrum and milk from cows at various stages of lactation showed that the ratio was lowest at the end of the first week post partum and that it increased gradually thereafter.

(5) Thyroxine increased ester-phosphorus markedly. It also increased lipid-phosphorus and caused a transient increase in casein-phosphorus. These increases were partly at the expense of inorganic phosphate which was decreased. Thiouracil affected the partition of phosphorus in the opposite way. The ester-phosphorus in milk was also found to be highest in the first week of lactation and lowest in late lactation, while inorganic phosphorus changed in the opposite direction.

(6) As a result of changes in the phosphatase and the partition of phosphorus, there were negative correlations between phosphatase on the one hand and ester-phosphorus and lipid-phosphorus on the other. Inorganic phosphate was positively correlated with phosphatase. Large negative correlations between

phosphatase and ester-phosphorus were found in the milk of untreated cows and in that of cows injected with thyroxine or thiouracil. The coefficients became significantly larger under the influence of either drug. These correlations were also found to hold in milk from cows at different stages of lactation.

(7) Thyroxine did not affect the total aneurin content of milk but thiouracil made a small but statistically significant decrease. Total aneurin in cows' milk was also found to decrease rapidly during the first few days of lactation, but the decreases became slower as lactation progressed.

(8) Thyroxine increased markedly the cocarboxylase content of milk, while a small but statistically significant increase also occurred in the protein-bound aneurin fraction. These increases were mostly at the expense of free aneurin which was decreased by thyroxine injection. Thiouracil changed the partition of aneurin in the opposite way. Cocarboxylase in colostrum of the first few days and in milk of early lactation was higher than in milk of late lactation. In late lactation the aneurin in the milk was almost exclusively in the free form.

(9) As the changes of phosphatase were in the opposite sense to those of the phosphorylated forms of aneurin, a close negative correlation between the phosphatase and cocarboxylase was recorded. Likewise there was also a close negative correlation between phosphatase

and protein-bound aneurin. The correlation of phosphatase with free aneurin was positive. These correlations were found in the milk of control cows, of cows injected with thyroxine or thiouracil, and of cows at various stages of lactation.

(10) The implications of the negative correlations between phosphatase on the one hand and various phosphoric esters on the other have been discussed. It is suggested, in the light of these experiments, that the chief effect of thyroxine may be to increase the concentration of phosphorylating enzymes in the tissues and so cause the well-known increase in the metabolic rate.

(11) It is concluded that in milk, low phosphatase concentrations are always associated with larger concentrations of phosphorylated esters and vice versa.

CHAPTER II

The effect of thyroxine and thiouracil on the concentration of the major constituents and on some of the water-soluble vitamins in cow's milk

Numerous investigators have tried to find whether treatment of lactating animals with thyroxine had any effect on the composition of the secretion. Almost invariably milk yield is markedly stimulated (Graham, 1934a, b; Jack & Bechdel, 1935; Folley & White, 1936; Herman, Graham & Turner, 1938; Smith & Dastur, 1940; Owen, 1948a). Usually the yield of fat is stimulated to a greater extent to yield of milk so that increased fat percentage results. This latter effect is, however, not always attained as may be seen by comparing the results of Smith & Dastur (1940) with those of Owen 1948a) in which the experiments were carried out with cows from the same herd. In some experiments the solids-not-fat of the milk were found to increase (Folley & White, 1936) but this again is not a constant finding (Archibald, 1945). Blaxter (1945) pointed out that this discrepancy in the magnitude of the change in solids-not-fat has sometimes been due to errors in calculation when concomitant changes in fat percentage were not taken into account. He showed that the concentration of non-fatty solids in the milk was increased by thyroxine provided that the calculations were made on the fat-free milk basis. The effect of feeding iodinated casein for increasing the milk and fat yield have been

investigated notably by Blaxter (1945, 1946) in commercial herds in England and Wales. The existing knowledge on the effect of iodinated casein and of thyroxine on milk yield and the composition of the secretion has been adequately reviewed recently by Blaxter, Reineke, Crampton & Petersen (1949).

The changes in the nitrogen content of milk caused by feeding iodinated casein or by treatment with thyroxine are highly variable. Thus Ralston, Cowsert, Ragsdale, Herman & Turner (1940) have reported a decline in the nitrogen content of milk. On the other hand Van Landingham, Hyatt & Weakley (1946), Hibbs & Krauss (1947) and Owen (1948a) found no change in the nitrogen or protein content of milk.

Until the present investigation was made no remarkable changes caused by thyroxine had been reported for minor constituents of milk except by Owen (1948b) who found a significant increase in the total phosphorus content of milk. It has been shown in Chapter I of the present thesis that the changes in the partition of phosphorus were more dramatic than any changes reported so far. These effects are definitely attributable to the thyroid gland because reverse changes have been shown to take place by administration of the antithyroid drug, thiouracil. In this chapter the results for some of the constituents not dealt with in Chapter 1 are briefly presented. In spite of the wealth of information available on the effect of thyroxine and iodinated casein on the proximate composition of milk, comparatively little

work has been done on the effect of hypothyroidism on milk composition. The original experiment of Graham (1934a) on the effect of thyroxine in increasing the milk and butter fat production arose from the observation that thyroidectomy inhibited milk secretion. In the present study the effects of administering thiouracil on milk composition were simultaneously studied as a negative control to that of thyroxine treatment. Results for the effect of thyroxine and thiouracil on the riboflavin and ascorbic acid content of milk are also recorded in this section.

Experimental

The analyses of milk were carried out on the same cows as described in experiments 1 - 3 in Chapter I. Milk samples were obtained every second day throughout the experiments. Period 2 was the treatment period, and Periods 1 and 3 were control periods. Each period lasted three weeks. Some cows were kept as untreated controls, i.e. they did not receive any treatment even in Period 2. It was thus possible to compare the composition of milk during treatment with that of the milk secreted by the same cow before and after treatment and also with the milk of the untreated cow secreted at the same time.

Methods of analysis

The milk samples have been analysed by standard procedures. Fat was determined by the Gerber method (British Standards Institution, 1936), total solids by drying a weighed sample of the milk first in a water-bath

and then in a thermostatic oven at 100°. The estimation of total nitrogen has already been referred to in Chapter I. Protein was calculated by multiplying the total nitrogen by the factor 6.38. Lactose was determined polarimetrically by Vieth's method as described by Elsdon & Walker (1942), the necessary corrections being made for the volume of the precipitate. Chloride was estimated by the method of Davies (1938) and the freezing point depressions by an improved Hortvet apparatus described by Temple (1937). For estimating calcium, milk samples were evaporated in vitreosil basins first in a water-bath and then ashed in an electric muffle furnace which was thermostatically controlled at 600°. The ash was dissolved in warm 6N. HCl and made up to a known volume. The final stage in the estimation was the titration of precipitated calcium oxalate with $KMnO_4$. Details are given by Hawk, Oser & Summerson (1947).

Ascorbic acid was estimated by the method of Mattick, Hiscox, Crossley, Lea, Findlay, Smith, Thompson, Kon & Egdell (1945). Protein was precipitated from milk by adding an equal volume of a mixture of equal parts of 10% sodium metaphosphate and 8% trichloroacetic acid. The filtrate was titrated with a standard solution 2:6 dichlorophenol indophenol blue. The dye solution was standardised by titration of a solution containing a known quantity of pure synthetic ascorbic acid.

Riboflavin in milk was estimated by the method of Emmerie (1938). Methanol (50 ml.) was added slowly with stirring to 50 ml. skimmed milk. The mixture was

kept at 60° for 15 min. After cooling to room temperature, 0.1 ml. of glacial acetic acid was added and the volume made up to 100 ml. The mixture was then shaken and allowed to stand for 15 min. and filtered. A 70 ml. portion of the filtrate was taken in a flask and the volume was reduced to about 20 ml. at reduced pressure. Glacial acetic acid (0.5 ml.) and 1 ml. of 4% $KMnO_4$ were added. After 1 min. 1 ml. 3% H_2O_2 was added. The volume was made up to 25 ml. and the riboflavin concentration measured in the fluorimeter. Care was taken to prevent access of sunlight during this estimation.

Results and Discussion

Milk Yield

Graphs showing the increase in milk yield brought about by thyroxine and the decrease brought about by thiouracil are recorded in Chapters I and III (See Figs. 4 & 18). In this section the responses due to the drugs in terms of quantity of milk will be dealt with for all the cows used in the present series of experiments. For this purpose the results of nine control cows, nine cows treated with thyroxine and nine cows treated with thiouracil have been analysed statistically. The cows have been numbered 1 to 27 in addition to recording them by their names. This has been done to avoid confusion resulting from the same cow being used one year as control and treated with a drug in a later experiment.

In calculating the positive or negative responses in milk yield, it is obviously difficult to estimate

what the yield would have been had no treatment been given, since after the first few weeks post partum milk yield gradually declines. The increases caused by thyroxine and the decreases caused by thiouracil are therefore complicated by this natural decline. In order to get over this difficulty the average daily yield (x) in each cow during period 1 was analysed by covariance with the average daily yield (y) in period 2, the cows being divided into three treatment groups - controls, thyroxine and thiouracil. From the total sum of squares and products, the variance due to treatment was elimina ted. From the error line, a significant regression was established. The regression coefficient was found to be +0.94077 lb. or the expected yield in period 2 was 0.94077 times the initial yield plus a constant. This constant was calculated to be -0.44 lb. or in other words the regression line passed just below the origin. The expected yield (lb./day) in period 2 was therefore calculated for each cow from the equation y (lb./day) = -0.44 + 0.94077 x. The yields estimated in this way for period 2 are shown in Table 20 in which the actual yields have also been recorded. It can be seen that in the control cows the expected yields calculated from the regression line were comparable with the actual yields. There were slight discrepencies in individual animals but the mean for all the cows was in close agreement. The results for all the thyroxine cows except No.24 showed that the actual yield was higher than the expected yield. In contrast the actual yield in the

thiouracil cows again with one exception (No.26) were all lower than the expected yield. It will be observed from Table 20 that the percentage increase in milk yield caused by thyroxine over a period of three weeks treatment varied considerably between cows. Similarly thiouracil also caused highly variable negative responses. The initial yield of the cow did not seem to have any effect on the magnitude of the response caused by either thyroxine or thiouracil. The mean response in the thyroxine group was +13.6% and that in thiouracil group -9.8%.

Fat, solids-not-fat and protein

The percentages of fat and solids-not-fat (S.N.F.) are recorded in Table 21. The results of S.N.F. have been calculated on the fat free basis. It will be observed that in most animals thyroxine caused an increase in the fat content of the milk. The effect of the hormone on the S.N.F. content of milk was variable. In some cows there was a small increase, but in others there was no change. This contrast is particularly noticeable in the cow Dewdrop (Experiment 3) in which a maximum response in the fat content of milk was recorded without any change in S.N.F. Thiouracil decreased the fat content of milk except in the animal Trixie (Experiment 2) for which the fat content of milk was considerably increased in successive periods. The effect of thiouracil on the S.N.F. content of milk was also incânclusive though a small decrease was noticed in some cases. It therefore appears

that in general thyroxine caused an increase in fat and S.N.F. and that thiouracil caused a decrease in fat. With both drugs the effect on fat percentage was more marked than that on the percentage of S.N.F.

The protein content of the fat-free milk in the cows used in experiments 2 and 3 is recorded in Table 22. The results confirm the observations already discussed in Chapter I for total N in experiment 1. The values in Table 22 do not give any indication that the drugs had any significant effect.

Lactose, chloride and freezing-point

The results for lactose, chloride and freezing-point are recorded in Table 23. These analyses were carried out only in experiments 1 and 2. The results show that corresponding to a small increase in S.N.F. (Table 21) there was a small increase in the lactose content of milk but the effect was not noticeable in all the treated cows. Thiouracil caused a small decrease in lactose content corresponding to a decrease in S.N.F. The results for lactose content in the control cows show further that with the progress of lactation the lactose content was gradually decreased. This was in contrast to the protein content which was gradually increased as lactation progressed. Simultaneously with an increase in lactose content, there was a decrease in the chloride content of the milk in the thyroxine treated cows. Thiouracil had the opposite effect. The increase in chloride with a corresponding decrease in lactose was

also noticeable in the control cows with the progress in lactation. There was thus a close negative correlation between lactose and chloride. Davies (1936) pointed out that since lactose and chloride account for 80% of the osmotic pressure of milk, there tends to be a constant relationship between those two constituents. He found from analysis of numerous milk samples that the relationship can be expressed as

lactose content = 6.26 - 13.5 x chloride content

or in other words lactose plus 13.5 times chloride should be a constant 6.26. In the present investigation this constant has been calculated and recorded in Table 23 where it is referred to as the lactose-chloride number. It was found that this number was fairly constant for all the cows in all the periods but the actual value was somewhat lower than that of Davies (1936). The mean value in these experiments was found to be 6.19. In conformity with this constancy of the relationship of lactose and chloride it was also found that the freezing-point depression of milk remained unchanged throughout the experiments. The drugs had therefore no effect on the freezing-point of the milk.

Calcium. The result of calcium content of milk recorded in Table 24 show no change attributable to hormonal effect. They confirm the earlier observation of Owen (1948b) who did not find any change in the Ca content of milk as a result of thyroxine treatment. In conformity with this finding is the new observation that Ca is not

affected by thiouracil. The failure of hormone administration to change the calcium content of milk is in sharp contrast to the effect on the phosphorus content which was increased by thyroxine and decreased by thiouracil (p.28). This can perhaps be correlated, as pointed out earlier, with the low requirement of calcium by the soft tissues. These observations in conjunction with that of Owen (1948b) indicate that the metabolism of calcium and that of phosphorus in adult animals are not so directly linked as is generally supposed.

The sodium, potassium and magnesium contents of the milk were also estimated. Standard procedures were used. There was no indication that either drug caused any change. The results are therefore omitted for the sake of brevity. Dr P.S. Watts also analysed the blood of the cows in experiment 3 for haemoglobin, red blood cells and white blood cells. The daily variations were in all cases much higher than any changes likely to be produced by the drugs. It was therefore concluded that both thyroxine and thiouracil were without effect.

Watersoluble vitamins

(i) Riboflavin. The mean riboflavin contents of the milk in six cows (Experiment 3) are recorded in Table 25 with the standard errors. The normal day-to-day variations were so large that no definite conclusion could be drawn. The mean results of the two thyroxine treated cows showed a decrease in the treatment period (Period 2) compared with the pre-treatment period (Period 1) but this also occurred in one of the control cows and in the two

thiouracil cows. The results were therefore analysed statistically. The analysis of variance also recorded in Table 25 showed that the differences between cows were significant, but that the difference between periods within cows were not. The mean values in period 1 showed a range of 83 - 127 µg. per 100 ml. fat-free milk. From the analysis of variance it can therefore be concluded that neither the effect of the drugs nor the stage of lactation caused any change on the riboflavin content of the milk. Bartlett, Rowland & Thompson (1949) reported a 17% decrease in riboflavin in the milk of cows treated with thyroxine. The Arizona workers (Kemmerer, Bolomey, Vevich & Davis, 1946) surprisingly enough found a decrease, the concentration of riboflavin during the treatment period dropping almost to nil. These values are impossibly low and as Kon & Henry (1949) pointed out must have been due to some mistake. In view of the finding of Bartlett, Rowland & Thompson (1949) the present results of 2-day values were further analysed statistically by comparing the rates of decreases in the three groups of cows during period 2. The differences of the three regressions were found to be not statistically significant. Thompson (quoted by Kon & Henry, 1949) also found no effect on the riboflavin content of milk by treating cows with thyroxine or iodinated protein.

(ii) Ascorbic acid. In determining ascorbic acid two precautions were taken throughout. The samples of milk were always made large enough to fill the bottle to

prevent any aerial oxidation and milk was pipetted into the trichloroacetic acid-metaphosphoric acid mixture within 15 min. of the collection of the samples to avoid formation of any dehydroascorbic acid. The results are illustrated in Fig. 13 for the three cows in Experiment 1, and in Fig.14 for the five cows in Experiment 2. It will be observed that in all the thyroxine treated cows, the ascorbic acid content decreased during the treatment period while in the thiouracil cows it increased steadily throughout the treatment. The control cows remained fairly constant throughout the experiments, showing that the stage of lactation had little effect on the ascorbic acid content of the milk. The numerical mean values of all the thyroxine and thiouracil treated cows (Experiments 1, 2 & 3) before and during treatment are recorded in Table 26. The percentage responses have also been calculated and shown in the Table. It will be observed that the mean decrease caused by thyroxine was 25% (range 14 - 34%) while the mean increase in the cows receiving 20 mg. thiouracil was 24%. It can also be noticed that the decrease was only 10% in the cow dosed with 10 mg. thiouracil. Decreases in ascorbic acid content of milk after treatment of cows with iodinated casein have been recorded by Van Landingham, Henderson & Weakley (1944) and by Bartlett, Rowland & Thompson (1949). The latter authors attributed this effect to iodine present in the iodinated casein. The present results, however, indicate that this effect is due to the hormone, because the changes were in the opposite

Fig 13 Effect of thyroxine and thiouracil on the ascorbic acid content of the cow's milk. (Expt I.)

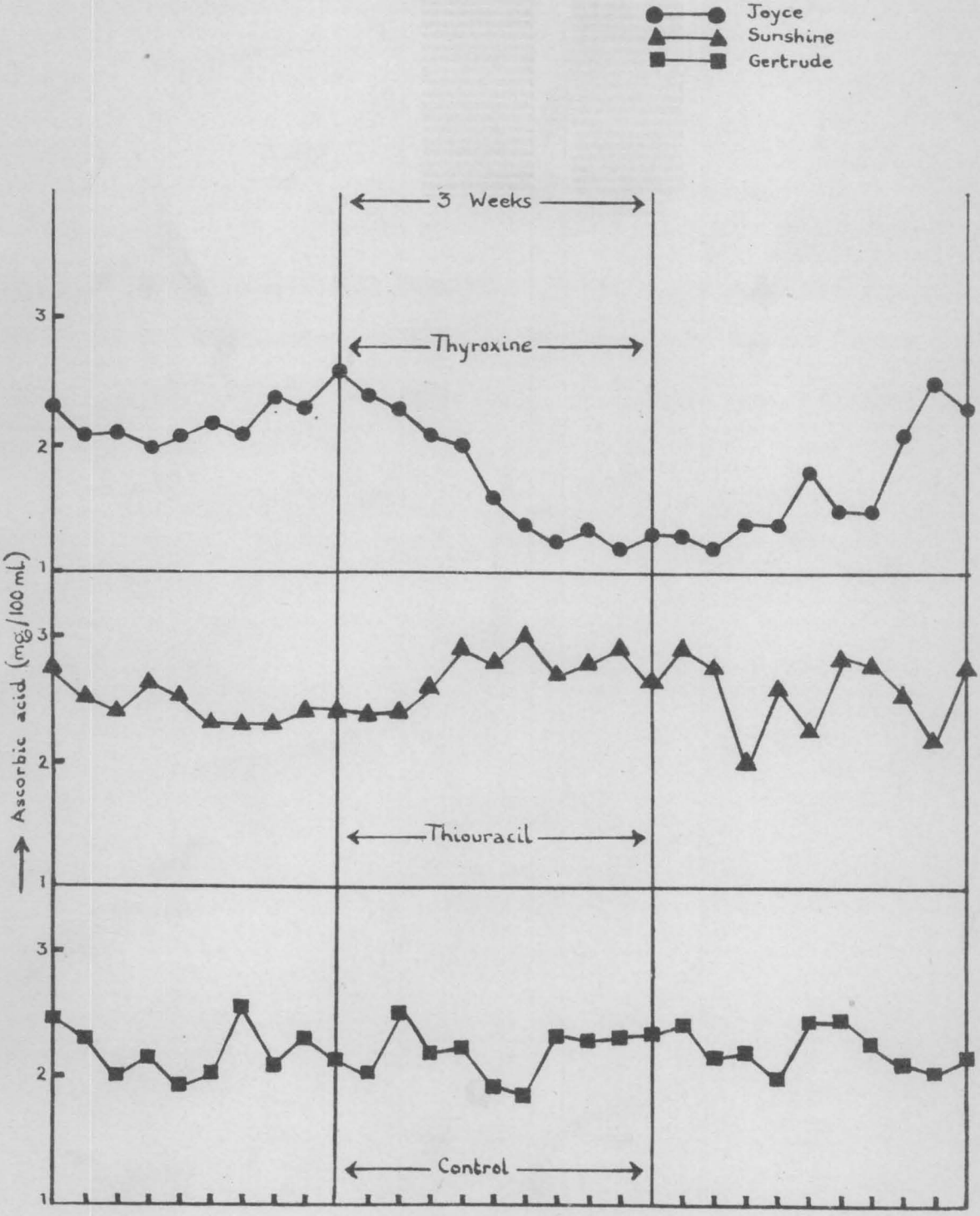
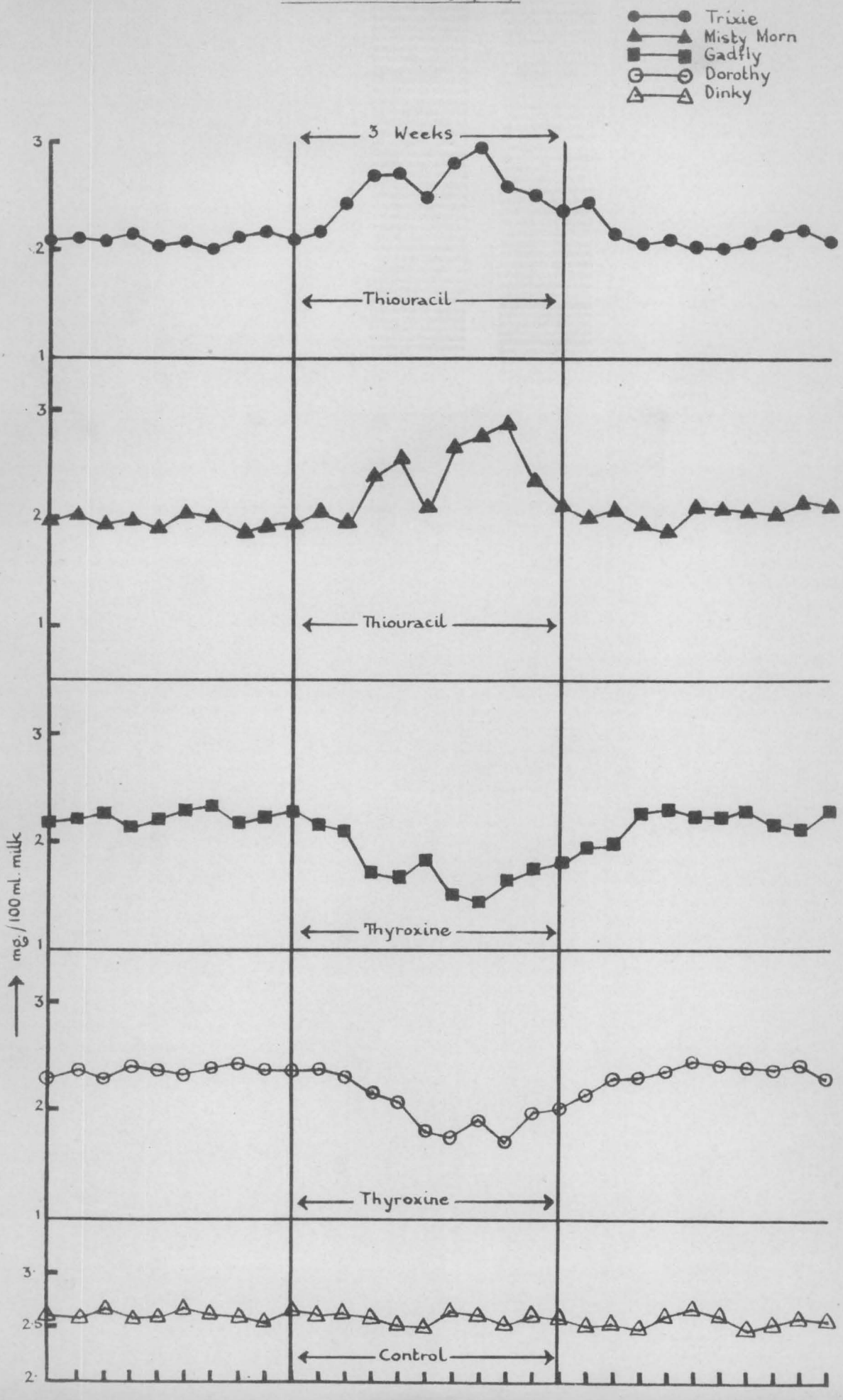


Fig. 14 Effect of thyroxine and thiouracil on the ascorbic acid content of the cow's milk. (Expt. 2)



direction in the cows receiving thiouracil and because the amount of iodine given in 10 mg. thyroxine per day could not in itself be expected to influence the ascorbic acid content of the milk. There is some indication that thyroid probably governs the ascorbic-acid content of milk. Kothavalla & Singh Gill (1943) reported that in India the concentration of ascorbic acid in the milk of Ayrshire and cross bred cows was lower than in the Zebu cattle of Sindhi and Gir breeds. These variations can partly be correlated with the difference in the state of thyroid gland in the two species, Bos taurus and Bos indicus.

Summary

The effect of thyroxine and thiouracil on the composition of cows' milk has been investigated. The following are the main results.

(1) Thiouracil decreased milk yield in lactating cows. This effect was the reverse of the well-known effect of thyroxine in increasing the milk yield which was also confirmed in the present series of experiments. Thyroxine caused an increase of 13.6% in milk yield during three weeks of treatment, and thiouracil decreased the yield by 9.8%.

(2) Thyroxine caused an increase in the fat and in the solids-not-fat content of milk, and thiouracil caused a decrease. The effect on fat content was more marked and less variable than the effect on S.N.F. It was also found that neither of these two effects are always peproducible.

(3) The protein content of fat-free milk remained unchanged when the cows were treated with thyroxine or thiouracil.

(4) There was a small increase in the lactose content of milk in the thyroxine treated cows corresponding to a simultaneous decrease in the chloride content. Thiouracil caused a reverse effect in the constituents so that there was a constant relationship between lactose and chloride. The freezing-point of milk remained unchanged during treatment with the drugs.

(5) The calcium content of the milk was unaffected by either treatment. This was in sharp contrast with the phosphorus content which was increased by thyroxine and decreased by thiouracil.

(6) There was no change in the riboflavin content of the milk when the cows were treated with thyroxine or thiouracil.

(7) Thyroxine decreased the ascorbic acid content of the milk by 25%, while thiouracil caused an increase of 24%. When the dosage of thiouracil was halved, the increase was only 10%.

Table 20. The effect of thyroxine and thiouracil on milk yield

Group of cows	Cow	Expected milk yield during period 2 calculated from the regression (lb./day)	Actual milk yield during period 2 (lb./day)	Response (%)
Controls	1 Gertrude	11.31	10.99	-
	4 Dinky	10.50	9.86	-
	27 Anitra	12.18	11.78	-
	9 Sunshine	28.28	28.52	-
	10 Ella	32.14	32.00	-
	15 Dora	24.97	24.70	-
	16 Tinker	24.79	25.18	-
	21 Dora	22.87	23.31	-
	22 Tinker	22.81	23.96	-
Mean		21.09	21.14	
Thyroxine	2 Joyce	9.21	10.05	+ 9.1
	5 Dorothy	15.08	19.01	+26.1
	6 Gadfly	10.29	12.19	+18.5
	11 Gertrude	30.29	32.36	+ 6.9
	12 Dewdrop	23.85	26.19	+ 9.8
	17 Delilah	19.41	24.32	+25.3
	18 Griselda	15.17	18.48	+21.8
	23 Delilah	18.50	19.59	+ 5.9
	24 Griselda	12.02	11.93	- 0.8
Mean		17.09	19.35	+13.6
Thiouracil	3 Sunshine	20.01	18.84	- 5.8
	7 Misty Morn	20.38	16.73	-17.9
	8 Trixie	13.58	11.32	-16.6
	13 Jassamine	34.19	28.28	-17.3
	14 Jenny	32.02	29.25	- 8.7
	19 Jean	21.56	19.61	- 9.0
	20 Gwynneth	21.86	20.43	- 6.5
	25 Jean	14.75	13.43	- 9.0
	26 Gwynneth	17.27	17.74	+ 2.7
Mean		21.74	19.51	- 9.8

Table 21. The effect of thyroxine and thiouracil on the contents of fat and solids-not-fat in milk

Experiment	Cow	Treatment in period 2	Fat (g./100g. milk)			S.N.F. (g./100g. fat-free milk)		
			Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
1	Gertrude	None	4.07	3.75	4.15	8.78	8.89	9.15
	Joyce	Thyroxine	3.83	4.20	5.00	8.84	9.11	9.92
	Sunshine	Thiouracil	3.82	3.23	3.38	8.46	8.21	8.57
2	Dinky	None	4.45	4.34	4.78	8.86	8.87	9.20
	Dorothy	Thyroxine	4.51	5.08	4.83	9.45	9.96	9.61
	Gadfly	Thyroxine	4.31	4.43	4.61	8.75	8.63	8.97
	Misty Morn	Thiouracil	4.06	3.62	3.46	8.48	8.53	8.52
	Trixie	Thiouracil	4.19	4.66	5.13	8.79	8.83	9.33
3	Sunshine	None	3.95	3.90	4.24	8.92	8.89	8.78
	Ella	None	4.28	3.95	3.98	8.88	8.81	8.54
	Gertrude	Thyroxine	4.12	4.74	4.35	9.24	9.43	8.95
	Dewdrop	Thyroxine	4.14	5.24	4.74	9.01	8.97	8.72
	Jassamine	Thiouracil	4.44	4.12	4.58	9.05	8.85	8.76
	Jenny	Thiouracil	3.91	3.47	4.32	9.02	9.01	8.84

Table 22. The effect of thyroxine and thiouracil on the protein content of milk

Experiment	Cow	Treatment in period 2	(g./100g. (± S.E.) fat-free milk)		
			Period 1	Period 2	Period 3
2	Dinky	None	3.59 ± 0.25	3.87 ± o.31	4.25 ± 0.48
	Dorothy	Thyroxine	3.87 ± 0.37	3.94 ± 0.37	3.93 ± 0.35
	Gadfly	Thyroxine	3.67 ± 0.28	3.50 ± 0.34	3.97 ± 0.42
	Midty Morn	Thiouracil	3.38 ± 0.23	3.22 ± 0.21	3.25 ± 0.34
	Trixie	Thiouracil	3.65 ± 0.32	3.99 ± 0.34	3.95 ± 0.29
3	Sunshine	None	2.85 ± 0.29	2.83 ± 0.36	2.91 ± 0.34
	Ella	None	2.92 ± 0.36	2.85 ± 0.24	2.92 ± 0.28
	Gertrude	Thyroxine	3.27 ± 0.31	3.21 ± 0.27	3.05 ± 0.25
	Dewdrop	Thyroxine	3.00 ± 0.27	3.11 ± 0.39	3.06 ± 0.34
	Jassamine	Thiouracil	3.00 ± 0.24	3.05 ± 0.29	2.99 ± 0.36
	Jenny	Thiouracil	2.84 ± 0.25	2.87 ± 0.21	3.03 ± 0.42

Table 23. The effect of thyroxine and thiouracil on the lactose and chloride contents of milk

Experiment	Cow	Treatment in period 2	Period	Lactose (%)	Chloride (%)	Lactose-chloride number*	Freezing-point (Δ)
1	Gertrude	None	1	4.13	0.151	6.17	0.545
			2	4.09	0.155	6.18	0.541
			3	4.05	0.158	6.18	0.542
	Joyce	Thyroxine	1	4.22	0.145	6.18	0.546
			2	4.47	0.125	6.16	0.539
			3	4.16	0.152	6.21	0.544
	Sunshine	Thiouracil	1	4.33	0.137	6.18	0.538
			2	4.06	0.156	6.17	0.542
			3	4.37	0.139	6.25	0.546
2	Dinky	None	1	4.39	0.132	6.17	0.542
			2	4.21	0.148	6.21	0.539
			3	4.09	0.155	6.18	0.541
	Dorothy	Thyroxine	1	4.67	0.112	6.18	0.545
			2	5.18	0.076	6.21	0.546
			3	4.76	0.105	6.18	0.542
	Gadfly	Thyroxine	1	4.28	0.144	6.22	0.539
			2	4.32	0.138	6.18	0.542
			3	4.11	0.154	6.19	0.537
	Misty Morn	Thiouracil	1	4.25	0.142	6.17	0.547
			2	4.29	0.140	6.18	0.541
			3	4.35	0.139	6.23	0.545
	Trixie	Thiouracil	1	4.31	0.138	6.17	0.551
			2	4.01	0.159	6.16	0.547
			3	4.44	0.135	6.26	0.549

*Lactose + 13.5 chloride = constant (6.26) (Davies, 1936)

Table 24. The effect of thyroxine and thiouracil on the calcium content of milk

Cow	Treatment in period 2	(mg./100 g. ± S.E.)		
		Period 1	Period 2	Period 3
Sunshine	None	118.9 ± 2.8	120.4 ± 3.2	109.0 ± 2.9
Ella	None	123.4 ± 3.7	109.1 ± 3.6	111.5 ± 3.2
Gertrude	Thyroxine	133.7 ± 2.4	132.7 ± 3.2	119.3 ± 3.5
Dewdrop	Thyroxine	139.7 ± 3.6	128.8 ± 2.9	123.0 ± 2.5
Jassamine	Thiouracil	125.2 ± 2.5	117.7 ± 2.8	119.6 ± 2.6
Jenny	Thiouracil	123.5 ± 3.1	119.9 ± 3.2	116.9 ± 2.9

Table 25. The effect of thyroxine and thiouracil on the riboflavin content of cow's milk

Treatment in period 2	Cow	(μg./100 ml. fat-free milk)		
		Period 1	Period 2	Period 3
Thyroxine	(Gertrude	111.5 ± 2.8	97.5 ± 3.1	108.1 ± 3.6
	(Dewdrop	103.0 ± 1.5	95.1 ± 2.9	100.2 ± 3.4
None	(Sunshine	103.2 ± 1.6	105.4 ± 2.2	102.2 ± 4.2
	(Ella	82.5 ± 1.7	77.2 ± 2.9	77.0 ± 2.4
Thiouracil	(Jassamine	104.6 ± 1.6	103.5 ± 2.6	99.5 ± 4.1
	(Jenny	127.2 ± 2.2	115.7 ± 2.5	107.0 ± 4.2

Analysis of variance of the average results for riboflavin

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Total	17	2687.58	-	-
Cows	5	2302.18	460.44	20.39***
Periods	2	159.61	79.81	3.53 (N.S.)
Error	10	225.79	22.58	-

Table 26. The effect of thyroxine and thiouracil on the ascorbic acid content of cows' milk

Treatment	Cow	Ascorbic acid (mg./100 ml. milk)		Response (%)
		Initial period	Experimental period	
10 mg. thyroxine/ day	Joyce	2.21	1.70	- 23.1
	Dorothy	2.36	2.02	- 14.4
	Gadfly	2.23	1.77	- 20.6
	Gertrude	2.13	1.43	- 32.9
	Dewdrop	2.45	1.61	- 34.3
10 mg. thiouracil /day	Sunshine	2.46	2.70	+ 9.8
20 mg. thiouracil /day	Misty Morn	1.97	2.40	+ 21.8
	Trixie	2.10	2.59	+ 23.3
	Jassamine	2.36	2.99	+ 26.7
	Jenny	1.99	2.51	+ 26.1

Chapter III

The Metabolism of Carotenoids and Vitamin A in Lactating Cows and Goats, with Special Reference to Nutritional and Hormonal Effects on the Secretion of Vitamin A in their Milk

Object

The carotene intake of lactating animals is one of the most important factor influencing the vitamin A potency of the butter produced from their milk. The sole source of vitamin A for the ruminant is the carotene in the feedingstuffs. This it converts to vitamin A and secretes in milk partly as vitamin A and partly in the original form. The relative proportions secreted as carotene and as vitamin A vary largely between species and between breeds of the same species. The intergeneric differences in the ratio of carotene to vitamin A in Bovidae are greater than intrageneric. Watson, Bishop, Drummond, Gillam & Heilbron (1934) reported that there was extremely little carotene (uncorrected for xanthophyll) in goat's milkfat compared with the amount in cow's milkfat, and yet the vitamin activity in the goat fat was the higher. Control cows in mid January secreted 292 μg/100 g. dry butterfat while goat butter examined in December contained 450 μg./100 g. dry butterfat. Hvidsten, Harsteen & Broch (1948) recently investigated the content of vitamin A in milk from goats and cows at pasture. They also noticed the presence of only traces of carotene in goats' butter while the total vitamin A activity was greater in the goats' milk than in the cows' milk.

The present investigation was designed to study the absorption of carotene in ruminants when grass meal constituted about a quarter of the concentrate mixture in a winter ration. The efficiency with which the ingested carotene was converted to vitamin A in the milk was determined. The transfer of carotene to the milk of cows and goats was also measured. The main object was, however, to investigate more critically whether the efficiency of absorption and secretion is influenced by subcutaneous administration of thyroxine or thiouracil. As a prerequisite a fundamental study was made of the usefulness of Cr_2O_3 as an indicator in studies of carotene metabolism in ruminants. The chapter has therefore been divided into two sections. Section 1 deals with the use of chromium sesquioxide (Cr_2O_3) to measure the digestibility of carotene by the goat and the cow, while Section 2 deals with the effect of thyroxine and thiouracil on the absorption of carotene and on the secretion of vitamin A activity in milk. Some typical figures illustrating the vitamin A activity present in cows' and goats' milk under the winter feeding conditions prevailing at the Hannah Institute are reported. The effect of feeding a carotene-free diet to lactating cows was also studied.

Section 1

The use of chromium Sesquioxide to measure the digestion of carotene by the goat and the cow

In determining the digestibility of any nutrient the estimation of the complete daily output of faeces by large animals is very laborious, requires careful surveillance and often entails severe restriction of freedom of the animal investigated. Great technical simplification and considerable reduction of labour can be achieved by using internal markers for food and faeces. If x is the ratio of a given nutrient to the marker in the food and y the ratio of the same nutrient to the marker in the faeces then the digestibility of the given nutrient is $100 \frac{(x-y)}{x}$ %. The theoretical requirements for success of such a method are that the marker should be intimately mixed with the nutrient, the digestibility of which is under test, and that this intimacy of mixing should persist throughout the alimentary tract. It is further necessary that whatever marker is used, it should be readily determined chemically, that it should not be present in foodstuffs in more than traces, and that none of the marker should be absorbed by the animal. It is in respect of these theoretical requirements that the use of natural constituents of foods such as lignin or silica is open to objection, in spite of the fact that in certain experiments such natural markers have been shown to give accurate results.

The use of various markers in digestibility experiments has been reviewed by Kane, Jacobson & Moore (1950) and by Owen (1951). With lignin, silica or iron there is a considerable chance that a sample of food chosen for analysis on a given day will differ in its content of these substances from the supposedly comparable sample actually fed on that day. Silica suffers an additional disability as a marker, for if any of it is present as particles of sand in the leaf-sheaths of cereals or hay it will tend to accumulate in the rumen or caecum or in any other part of the gut where the rate of passage of food is slowed down. Indeed post-mortem examination frequently shows sand in the rumen.

Chromium sesquioxide which was introduced by Edin (1918, 1926), is the indicator of choice for none of the objections stated above applies to it. According to Grushko (1948) and de Saint-Rat (1948) it occurs in even the richest plant materials in a concentration of less than 0.5 parts per million of dry matter, and only traces of it are absorbed by animals. Unlike iron it is not widely distributed in great concentration on the earth's surface, being confined to "ultra basic" rocks. Chance contamination with it is therefore not a hazard. Chromium sesquioxide is used commercially as a pigment and is obtainable in the form of an impalpably fine powder which readily remains in suspension in the contents of the gut.

Chromium oxide has been successfully used as a marker by Edin (1918, 1926), Hamilton, Mitchell, Kick & Carman (1927-28), Andersen (1934), Andersen & Frederiksen (1935), Jarl (1946), Skulmowski, Szymanski & Wyszynski (1943), Kreula (1947), Kane, Jacobson & Moore (1950) and Schurch, Lloyd & Crampton (1950).

The present writer was interested in the effect of thyroxine and thiouracil on the fate of carotene in ruminants and wished, among other things, to compare its fate in the cow, which secretes milk containing both carotene and vitamin A, with its fate in the goat, which secretes milk containing vitamin A but estremely little carotene. A necessary part of the experiment, therefore, was the determination of the 'digestibility' of carotene in the cow and the goat. For the sake of brevity the term 'digestibility' is used in this study to denote the percentage of the ingested carotene which was not excreted as carotene in the faeces.

$$\text{'Digestibility' of carotene} = \frac{\text{Amount of carotene ingested} - \text{amount of carotene excreted in faeces}}{\text{Amount of carotene ingested}} \times 100$$

In the cows the digestibility was determined by using chromium sesquioxide since a direct determination was not possible. From the goats, complete collections of faeces were made using crates specially designed for the purpose so that the digestibility of carotene by them was determined both by the direct method and by means of chromium. The object of the

present experiments was to demonstrate the agreement of figures for digestibility of carotene determined by the two different methods in the goat and hence by implication to establish the validity of figures for digestibility of carotene obtained by using the chromium method in the cows. Results of both methods were statistically analysed with a view to assessing sources of variability and some advantages of the marker over the direct method are discussed.

Experimental

The digestibility of carotene was determined on six cows and four goats all in milk. The ration for the goats consisted of 9 parts of oats, 6 parts of decorticated earthnut and 6 parts of dried grass meal. To 31.5 cwt of this mixture 2300 g. Cr_2O_3 was added. The author himself assisted with the mixing which was done at a local mill. The Cr_2O_3 was first mixed with the least bulky constituent - the earthnut. The resulting mixture was then mixed with the other constituents using large wooden shovels. At this stage the oats were still whole. To crush the oats the whole mixture was next put through the mill and during the grinding a thorough mixing of the Cr_2O_3 with the other constituents took place. The daily ration for each goat was 3 lb. of this mixture and 1 lb. of oat glumes (the winnowings from the threshing of fully ripened oats). The goats were fed this ration for several weeks before being

placed in specially designed metabolism crates. After allowing two weeks for the goats to become accustomed to being in the crates the metabolism trial proper was begun. All intakes were corrected for food refusals. For chromium and carotene analyses complete collections of faeces were made every two days and on alternate days samples of the food were taken.

In the experiment with cows six lactating animals were under test. Initially they were fed a carotene-free ration of oats, beans (Vicia faba), potato starch, blood-meal and oat straw which had been ground to a powder. Each animal received 21 lb. of this mixture and 10 lb. oat straw daily. Faeces samples (2 lb.) were collected in the byre between 9 a.m. and 11 a.m. every second day for a fortnight. The faeces became free of carotene after about five days. Thereafter the cows continued to receive the 10 lb. oat straw but the carotene-free mixture was replaced by 21 lb. of a mixture consisting of 9 parts oats, 6 parts beans and 6 parts dried grass meal and containing 2100 g. Cr_2O_3 in 31.5 cwt. The cows continued on this grass meal ration for 3 weeks during which the concentrate mixture and the faeces were sampled on alternate days, and analysed for carotene and chromium.

Chemical methods of analysis

Carotene in food and faeces was estimated by the method of Seshan & Sen (1942a) combined with chromatography. About 2 g. of food or 10 g. of faeces were

weighed out immediately after sampling in a wide-mouthed flask and 3 g. KOH added. A few ml. of water were added to make the sample sufficiently wet but care was taken to avoid excess. The sample was allowed to soak for 10 min. by rotating the flask and was then heated under an air reflux condenser on a boiling water-bath for 30 min. to disintegrate the tissues. 50 ml. of addehyde-free ethanol were then added, the contents of the flask mixed thoroughly by shaking and the boiling under reflux continued for another 30 min. The temperature of the water-bath was slightly lowered at this stage. The flask was allowed to stand overnight in a slanting position, and then the supernatant liquid was decanted off into a separating funnel. The residue was washed three times with 15 ml. portions of ethanol decanting the extract in the separating funnel. The residue was then washed three times with 20 ml. portions of light petroleum (B.P. 40-60), and the light petroleum extracts added to the mother liquor in the separating funnel. The combined extract was shaken and allowed to settle. When the light petroleum was cleanly separated (the separation was hastened by adding 20 ml. of water at this stage) the lower layer was drawn off in an Erlenmeyer flask and the light petroleum layer in another flask. The lower layer was transferred back in the separating funnel and the extraction process repeated three times with 40 ml. portions of light petroleum. The extracts were combined and washed three times with 70 ml. portions of distilled water to remove

alkali. After the extract was free from alkali it was drawn off into a conical flask containing about 5 g. anhydrous sodium sulphate. The separating funnel was washed twice with small volumes of light petroleum and the washings added to the extract. The extract was filtered through cotton wool into a 250 ml. flask. The sodium sulphate was washed twice with 10 ml. portions of light petroleum and the washings were filtered and added to the extract. The volume of the extract was then reduced to about 10 ml. by evaporation under reduced pressure. The reduced extract was chromatographed in a 5 x 1 cm. column of alumina and carotene was eluted with 30 ml. of 3% acetone in light petroleum. The volume was made up to 50 ml. and the extinction was measured at 451 mμ in a Unicam Spectrophotometer. Carotene was calculated assuming $E\ \frac{1\%}{1\ cm}$ to be 2500 (Morton, 1943).

After elution of the carotene the column was washed with 10 ml. light petroleum and made ready for a second sample. One column can be used for four to six samples. Suction was applied gently throughout the chromatographic process taking care that the top of the column never went dry. A little anhydrous sodium sulphate was used on the top of the column.

Chromium was estimated in food and dry faeces by the method of Sandell (1936) adapted for use with biological materials. A dry method of ashing was thought simpler than wet ashing because it has far fewer steps and involves no additions of corrosive liquids or

generation of acidic fumes. A sample of food or dried faeces weighing 10 g. was ashed in a vitreosil basin in an electric muffle, the temperature of which was adjusted to 500°. The resulting sample of grey ash was weighed and then ground with a pestle without removing it from the vitreosil basin. An accurately weighed portion of the ash (about 100 mg.) was transferred to a nickel crucible, mixed with five times its weight of anhydrous sodium carbonate, and fused for 20 min. in an electric muffle furnace at 900°. Ashings carried out in platinum and nickel showed that pure nickel as recommended by Hardwick (1950), could be used as a substitute for platinum. After it was cool, the ash was repeatedly extracted with hot water. The resulting green alkaline solution was heated at 100° with a few drops of ethanol in a 50 ml. pyrex beaker to remove manganate. The yellow solution remaining was filtered into a 50 ml. volumetric flask. The filter paper was washed with 1% Na_2CO_3 and the filtrate made up to 50 ml. The yellow chromate was read in a spekker photoelectric absorptiometer using a violet filter.

Results

The recovery of Cr in the goat experiment is recorded in Table 27. The results show that although recoveries varied considerably from one 2-day period to another, the total intake at the end of the whole experiment for each animal tallied well with the total output. The variation from period to period was duemainly to two

factors. These were variable food wastage (for which correction was made by collecting all refused food) and a variation of chromium content of the food due to difficulties of mixing. Table 28 shows the variation in the Cr and carotene contents of the samples of ration analysed. As would be expected from differences in the composition of the mixture from sample to sample, the variations of Cr were not in step with the variations of carotene. Table 27 shows the over-riding importance of adequate mixing in experiments with markers. In spite of the variation of recovery of Cr from one 2-day period to another, the overall recoveries for the whole ten days were 100, 99.1 and 99.1 per cent. for the goats, Anna, Betty and Bluebell respectively. In the goat, Diana, the recovery was only 96%. This goat, however, did not readily submit to being confined to the metabolism crate and was not a clean feeder, so that the estimation of refused food was difficult with this animal. Another source of variation shown in Table 28 is the gradual decline of the carotene content of the ration as the experiment proceeded. This was to be expected from analogy with the observations of Fraps & Kemmerer (1937) who found that carotene in grass-meal decomposed more quickly in the presence of starch than in grass-meal stored by itself.

The coefficient of variation of Cr content of the food was greater (c.v. = $\pm$ 16%) in the cow experiment than in the goat experiment (c.v. = $\pm$ 8.6%). The larger c.v. in the cow experiment was due to an un-

successful attempt to have the mixed ration made into "cubes", and perhaps also to the fact that the cow ration was the first one to be mixed with the Cr_2O_3. The goat ration was made after considerable experience had been gained regarding the most efficient methods of mixing.

The apparent digestibility of carotene in the goat experiment by both methods is shown in Table 29. The agreement between the results of the two methods was close, and the goat, Anna, which had a good appetite and was a good milker gave the best digestibility by each of the methods of testing. The agreement between the two methods was even better when the digestibility found by the direct method was corrected for the food refusals which escaped measurement but which were calculated from the incomplete recovery of Cr_2O_3. The results of this correction are shown in Table 30. The coefficients of variation of the average digestibilities obtained by the direct method and by the chromium method were $\pm$ 4.9% and $\pm$ 5.7% respectively. A detailed analysis of variance of the individual digestion coefficients by the two methods is shown in Table 31 in which the variances have been partitioned among the three variables - methods, goats and periods and their several interactions. The main conclusion from Table 31 is that the variability due to method was not significant, and that the main sources of variability were variations from goat to goat in any one period or from period to

period in any one goat.

The results for the cows (Table 32) show a range of digestibility of carotene by cows from 54.0% for Tinker to 59.3% for Dora. It is noticeable that for each cow the digestibility was highest in the first 2-day period. This is because just before the trial the cows had been eating (as described in the introduction) a diet free from carotene. Carotene in the cow therefore behaves ih the same way as other nutrients, e.g. N, Ca and P, in being more readily digested immediately after a period of deficiency. With the cows, as with the goats, there was a significant difference between the digestibilities from different periods (Table 33), but with the cows decline of digestibility from the beginning to the end of the experiment served in part to make this variance so significant.

Discussion

In these experiments Cr_2O_3 was mixed with the concentrate portion of the food, the roughages being left unmarked. The daily intake of carotene from the roughages was estimated to be only 0.1 mg. for the goats and only 1 mg. for the cows. This amount of carotene accounted for only about 0.2% of the intake and was ignored. If lignin or silica had been chosen as markers it would have been necessary to measure the ratio of carotene to lignin or silica in the roughages as well as the concentrates.

Where only the chromium method is used in experiments of this type an accurate knowledge of the ratio of carotene to chromium in the food is the most important factor necessary for calculating the apparent digestibility. This can be achieved either by mixing the chromium thoroughly with the carotene containing ration or by giving the chromium in capsules and keeping an accurate record of the feed consumption. In the present investigation the former course was adopted.

Part of the period to period variation on the recovery of Cr_2O_3 (Table 27) in the experiment with the goats, was due to the fact that a 2-days' output of faeces in a goat does not necessarily correspond very closely to a 2-days' intake of food. It therefore follows that collection periods need to be long enough to make this effect negligible. A lower recovery in any particular period was usually followed by a higher one in the next period, the division between periods being arbitrary. In the case of a ruminant this irregularity of defaecation is not unexpected since the passage of food is delayed in both the rumen and the caecum. One conclusion which can be drawn from both the goat and cow experiments is that a more reliable estimate of digestibility results from a longer experiment with fewer animals than from a shorter experiment with more animals. Jarl (1949), in replying to criticism by Eriksson (1949), drew a similar conclusion from his own experiments with Cr_2O_3. He also pointed out that the chromium method is more accurate than the direct method

because of the constant relation between the marker and the various nutrients in the ration and faeces, so that variation in the daily output of faeces between two consecutive periods does not affect the digestibility calculated. The corrected digestibility recorded in Table 30 supports this contention.

In these experiments goats proved more efficient digesters of carotene than cows. Thus Table 28 shows that the cows' ration contained 5.20 mg./100 g. carotene while the goats' ration contained 3.14 mg./100g. This lower concentration in the goat ration was purely accidental. It arose from the fact that the dried grass fed to the goats had a lower carotene content than that fed to the cows.

The average weight of the cows was 450 kg. and that of the goats 35 kg., and these figures were used to calculate the intakes of carotene per kg. body weight. Each cow consumed on the average 496 mg. carotene from 21 lb. of ration while each goat consumed only 42 mg. per day from 3 lb. of ration. Thus the intake of the cows was 1.10 mg. per kg. body weight while that of the goats was 1.20 mg. per kg. body weight. It follows therefore that in spite of almost equal intakes of carotene by the cows and the goats and in spite of the fact that the cows had been partially depleted by having had a ration free from carotene just prior to the digestibility trial, the goats were the better digesters. It may well be that the better digestion of carotene in the goat can be attributed to a more

active thyroid gland in that animal, for Schultze & Turner (1945) found that the rate of secretion of thyroxine by the lactating cow was 2.20 μg. per 100 g. body weight and that of the lactating goat 3.44 μg. per 100 g. body weight, and there is evidence for the rat that the thyroid governs the rate of accumulation and also the absorption of carotene (Cama & Goodwin, 1949a) and the storage of vitamin A in the liver (Johnson & Baumann, 1947). Experiments bearing on this aspect of hormonal effect on carotene absorption are reported in Section 2 of the present chapter.

Summary

The digestibility of carotene in dried grass was measured by the Cr_2O_3 method in both cows and goats. In the goats the direct method was simultaneously used for comparison with Cr_2O_3 method. The following were the main conclusions:

1. When mixed with the concentrate portion of the ration of lactating goats Cr_2O_3 was recovered in the faeces in four experiments in amounts equal to 100.0, 99.1, 99.1 and 96.0% of the amount ingested.

2. By the Cr_2O_3 method the digestibilities found for carotene in four goats were 67.4, 62.9, 61.8 and 58.9%. The corresponding digestibilities obtained from the same faeces by the direct method were 68.7, 62.7, 61.8 and 59.6%.

3. In six cows, on a diet similar to that of the goats, the Cr_2O_3 method gave the figures 59.3, 54.0, 54.4, 54.4, 57.1 and 55.5% for the digestibility of carotene.

4. Reasons for preferring the use of added Cr_2O_3 to natural constituents of the food as a marker are discussed.

5. The individual results from cows and goats were statistically analysed to partition the various sources of variance and it was shown that fewer animals for a longer time give a more reliable result than more animals for a shorter time.

6. It is suggested that the superiority in these experiments of goats over cows as digesters of carotene may be due in part to the goat's more active thyroid gland.

Table 27. Recovery in faeces of Cr_2O_3 in the diet of the goat

Goat	Period	Intake* (g. Cr/ 2 days)	Dry faeces voided (g./2days)	Cr in faeces (mg./ 100g.)	Cr excreted (g./2 days)
			(c)	(d)	$\frac{c \times d}{10^5}$
Anna	1	2.31	1206	193	2.33
	2	2.30	1276	152	1.94
	3	2.04	1390	195	2.71
	4	2.61	1172	204	2.39
	5	2.34	1009	222	2.24
	Mean	2.32	1211	193	2.32
Betty	1	2.24	1083	192	2.08
	2	2.29	1149	188	2.16
	3	1.91	1576	142	2.24
	4	2.58	1160	217	2.52
	5	2.32	1178	193	2.27
	Mean	2.27	1229	186	2.25
Bluebell	1	2.31	1332	166	2.21
	2	2.29	1494	160	2.39
	3	2.02	1665	128	2.13
	4	2.61	1347	161	2.17
	5	2.34	1272	202	2.57
	Mean	2.31	1422	163	2.29
Diana	1	2.26	946	245	2.32
	2	2.11	999	193	1.93
	3	1.96	1309	141	1.85
	4	2.58	1200	185	2.22
	5	2.34	983	252	2.48
	Mean	2.25	1087	203	2.16

* Corrected for refusals of food.

Table 28. The ratio of carotene to chromium (Cr) in the food of cows and of goats

Experimental animals	Period	Carotene in concentrate mixture (mg./100 g.)	Chromium in concentrate mixture (mg./100g.)	mg.carotene/g.Cr
		(a)	(b)	$(\frac{1000\ a}{b})$
	1	3.31	85	38.9
	2	3.18	84	37.9
Goats	3	3.12	75	41.6
	4	3.00	96	31.3
	5	3.10	87	35.6
	Mean	3.14 (c.v. = ± 3.6%)	85 (c.v. = ± 8.6%)	37.1
	1	5.49	79	69.8
	2	5.26	66	79.6
Cows	3	5.18	55	94.2
	4	4.85	77	63.0
	Mean	5.20 (c.v. = ± 4.4%)	69 (c.v. = ± 16%)	76.6

Table 29. The apparent digestibility of carotene in goats by the direct method and by the chromium method

Goat	Period	Carotene intake (mg./2 days)	Carotene excreted (mg./2 days)	Apparent digestibility (%) (Direct method)	mg.carotene/g. Cr in faeces	Apparent digestibility (%) (Chromium method)*
Anna	1	90.1	22.4	75.1	9.6	75.2
	2	86.5	26.8	69.0	13.8	63.5
	3	84.9	21.8	74.3	8.1	80.7
	4	81.6	39.8	51.2	16.7	46.7
	5	84.3	23.1	72.6	10.3	71.0
	Mean	85.5	26.8	68.4	11.7	67.4
Betty	1	87.2	30.2	65.4	14.5	62.7
	2	86.5	31.7	63.3	14.7	61.2
	3	79.6	32.3	59.4	14.4	65.3
	4	80.8	29.9	62.9	11.9	62.1
	5	82.6	29.9	63.8	13.2	63.1
	Mean	83.3	30.8	63.0	13.7	62.9
Blue-bell	1	90.1	34.4	61.9	15.5	60.1
	2	86.5	36.6	57.7	15.3	59.6
	3	84.0	21.5	74.4	10.1	75.8
	4	81.6	30.3	62.9	14.0	55.3
	5	83.5	38.2	54.3	14.9	58.3
	Mean	85.1	32.2	62.2	14.0	61.8
Diana	1	88.2	27.0	69.4	11.6	70.1
	2	79.7	31.4	60.7	16.3	57.0
	3	81.3	34.9	57.0	18.9	54.5
	4	80.8	35.0	56.6	15.8	49.5
	5	83.5	32.3	64.3	13.1	63.3
	Mean	82.7	32.1	61.6	15.1	58.9

* Note. If x represents mg. carotene/g.Cr. in the food of a given period (Table 28) and y represents mg.carotene/g.Cr in the faeces (Table 29) the digestibility expressed as a percentage of the carotene intake is:

$$100\left(\frac{x - y}{x}\right)$$

Table 30. Comparison of digestibility of carotene by the Cr_2O_3 method with the digestibility obtained by the direct method corrected for incomplete intake

Goat	Percentage Cr_2O_3 recovered (a)	Mean intake carotene (mg./2 days)* (b)	Corrected mean intake (axb/100)	Mean excretion	Corrected digestibility (Direct method)	Average digestibility (Cr_2O_3 method)
Anna	100.0	85.5	85.5	26.8	68.7	67.4
Betty	99.1	83.3	82.6	30.8	62.7	62.9
Bluebell	99.1	85.1	84.3	32.2	61.8	61.8
Diana	96.0	82.7	79.4	32.1	59.6	58.9

* Data from Table 29.

Table 31. Analysis of variance of apparent digestibility of carotene in goats

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Total	39	2325.86	-	-
(a) Methods (M)	1	11.03	11.03	a/g = 2.28 N.S.
(b) Goats (G)	3	328.44	109.48	b/g = 22.67*** b/f = 1.20 N.S.
(c) Periods (P)	4	760.13	190.03	c/g = 39.34*** c/f = 2.09 N.S.
(d) M x G	3	10.23	3.41	d/g = 0.71 N.S.
(e) M x P	4	67.27	16.82	e/g = 3.48*
(f) G x P	12	1090.77	90.90	f/g = 18.82***
(g) MxGxP (error)	12	57.99	4.83	-

N.S. not significant

* significant if $P < 0.05$

*** $P < 0.001$

Table 32. Apparent digestibility of carotene in cows using the chromium method

Cow	Period	Faecal carotene (mg./100g. dry faeces)	Faecal chromium (mg.Cr/100g. dry faeces)	mg.carotene /g.Cr	Apparent digestibility * (%)
		(a)	(b)	$(\frac{1000\ a}{b})$	
Dora	1	3.04	135	22.5	67.7
	2	4.78	148	32.2	59.5
	3	5.77	168	34.4	63.5
	4	4.36	130	33.7	46.5
	Mean	4.49	145	30.7	59.3
Tinker	1	3.15	120	26.2	62.5
	2	5.17	135	38.2	52.0
	3	6.34	139	45.5	51.7
	4	3.64	115	31.6	49.8
	Mean	4.58	128	35.4	54.0
Delilah	1	2.62	107	24.6	64.7
	2	5.67	147	38.6	51.5
	3	6.70	169	39.7	57.8
	4	3.85	108	35.7	43.4
	Mean	4.71	133	34.7	54.4
Griselda	1	2.96	115	25.9	62.9
	2	5.59	146	38.4	51.7
	3	6.19	161	38.5	59.2
	4	4.41	124	35.5	43.6
	Mean	4.79	136	34.6	54.4
Jean	1	2.92	116	25.1	64.0
	2	5.38	154	35.0	56.1
	3	5.83	178	32.7	65.3
	4	4.71	131	36.0	42.9
	Mean	4.71	145	32.2	57.1
Gwynneth	1	3.14	122	25.8	63.1
	2	5.62	160	35.2	55.8
	3	5.54	157	35.2	62.6
	4	4.10	110	37.4	40.6
	Mean	4.60	137	33.4	55.5

*Note. If x represents mg.carotene/g. Cr. in the food of a given period (Table 28) and y represents mg.carotene/g.Cr. in the faeces (Table 32) the digestibility expressed as a percentage of the carotene intake is $100(\frac{x - y}{x})$

Table 33. Analysis of variance of carotene digestibility in cows (Determined by the ratio method)

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	23	1550.83	-
Periods	3	1306.81	435.60 ***
Cows	5	85.56	17.11 N.S.
Error	15	158.46	10.56

N.S. not significant

*** significant if $P < .001$

Section 2

The effect of thyroxine and thiouracil on carotene metabolism in lactating cows and goats and on the secretion of vitamin A in their milk

Introduction

The inter-relationship of the thyroid and the metabolism of carotene and vitamin A has been investigated by various authors. Kunde (1926) observed the appearance of vitamin A deficiency in thyroidectomised rabbits, and Abelin (1933) noted defective carotenoid and vitamin A metabolism in hyperthyroid guinea pigs. Wendt (1935) observed a low serum vitamin A in patients with Graves' disease. When storage of vitamin A in the liver was used as a criterion of the efficiency of conversion of carotene to vitamin A in rats, Johnson & Baumann (1947) found that with identical doses of carotene, animals treated with thiourea stored less vitamin A than controls, which in turn stored less than animals receiving desiccated thyroid. The storage of vitamin A was normal when thyroxine was given at the same time as thiourea or thiouracil. Kelly & Day (1948) have confirmed this finding. Drill & Truant (1947), using remission of xeropthalmia as the criterion of conversion, failed to demonstrate the formation of vitamin A from carotene in thyroidectomised animals. In the light of recent interpretations the fact that these authors gave carotene parenterally is open to serious objection, for it now appears that the conversion of carotene to vitamin A takes place in the intestine (Glover, Goodwin

& Morton, 1948, Thompson, Ganguly & Kon, 1949). It should be mentioned, however, that Remington, Harris & Smith (1942) claim that eye symptoms in thyroidectomised rats are cured by oral administration of carotene, and that Di Bella (1940 a, b) making a similar observation noted a reduced efficiency of carotene utilisation in such animals.

Cama & Goodwin (1949 a) found that in rats thiouracil retarded the absorption of β-carotene from the intestinal tract of the rat and that desiccated thyroid stimulated it. The inhibitory effect of thiouracil was also counteracted by desiccated thyroid. Cama & Goodwin (1949b) could not substantiate the Russian claim (Kaplansky & Balaba, 1946) that thyroglobulin converts carotene to vitamin A in vitro. After 36 incubation experiments under varying conditions and after the study of the absorption spectra of the resulting products Cama & Goodwin suggested that the Russian workers may have attributed to vitamin A, absorption at 330 mμ which was due to the cisform of β-carotene resulting from isomerisation.

The effect of iodinated protein and of thyroidectomy on the carotene and vitamin A content of milk has been studied to some extent. Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) reported that the goat which they thought never to secrete any carotene in her milk, secreted yellow milk after thyroidectomy. Later work has failed to confirm this

report. Kon and his co-workers at Reading (Annual Report of the National Institute for Research in Dairying, 1944-45-46) failed to detect any carotene in the milk of thyroidectomised goats even when the animals were receiving 5 lb. carrots per day. Administration of thiourea was likewise ineffective. In a recent note Smith, Niedermeir & Schultz (1948) have also reported the failure to get yellow milk from hypothyroid goats. Reports on the effect of feeding iodinated protein are also conflicting. Bartlett, Rowland & Thompson (1949) found that it caused no change either in carotene or vitamin A in the fat of treated cows. In the work of Kemmerer, Bolomey, Vavich & Davis (1946) it appeared to cause no change in the vitamin A content of the milk, but Hibbs & Krauss (1947) claimed that feeding iodinated protein frequently decreased the vitamin A content of both the plasma and the milk.

The present investigations were carried out to make a systematic observation on the effect of subcutaneous injection of thyroxine and thiouracil on the secretion of vitamin A and carotene in the milk of cows and goats. The relation of this activity to the absorption of carotene was simultaneously investigated by metabolism experiments. The partition of vitamin A and carotene in milk was also studied when treatment with the drugs was superimposed on a carotene-free diet. The apparent digestibilities were determined by the direct method in the experiments with goats and by the Cr_2O_3 ratio method in the experiments involving cows,

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as outlined in Section 1 of the present Chapter.

Experimental

Two experiments were carried out. Six lactating Ayrshire cows were used in Experiment 1 and eight lactating goats in Experiment 2. At the beginning of the experiment the cows were from 6-10 weeks and the goats from 4-6 weeks from parturition.

Experiment 1 lasted for 16 weeks and was divided into seven periods as follows:-

riod No.	1	2	3	4	5	6	7
ration	2 weeks	3 weeks	2 weeks	3 weeks	3 weeks	2 weeks	1 week
ture of diet	Carotene-free (Diet 1)	Carotene (Diet 2)	Carotene-free (Diet 1)	Carotene (Diet 2)	Carotene (Diet 2)	Carotene-free (Diet 1)	Carotene-free (Diet 1)
	Hormonal treatments were superimposed on this dietary treatment as follows:-						
Cows							
inker & ora	None	None	None	None	None	None	None
elilah & riselda	None	None	None	10 mg. thyroxine daily to each animal	None	10 mg. thiouracil daily to each animal	None
ean & wynneth	None	None	None	20 mg. thiouracil daily to each animal	None	20 mg. thiouracil daily to each animal	None

The dietary regimes and the hormonal treatments are shown also in Figs. 15 and 16.

Diets 1 and 2 were made up to provide the same amount of starch equivalent (S.E.), digestible crude protein (D.C.P.) and fibre. The cows used were approximately of 1000 lb. live-weight and were giving about 3 gall. of milk daily at the start of the experiment. The fat content of the milk was between 3.9 - 4.2%. Maintenance requirements were assumed to be 6.0 lb. S.E. and 0.65 lb. D.C.P. per head per day, and the requirements per gallon of milk produced were calculated on the basis of 2.8 lb. S.E. and 0.60 lb. D.C.P. The total daily requirements were initially, therefore, 14.4 lb. S.E. and 2.45 lb. D.C.P. The cows each received 10 lb. oat straw and 21 lb. of a concentrate mixture daily. The composition of the diets with their S.E. and D.C.P. is shown in Table 34. A 21 lb. portion of either of the diets provided 13 lb. S.E. and 2.6 lb. D.C.P. The fibre contents of the two diets were the same. Although there was some initial variation in the milk yield from cow to cow and although fluctuations due to the treatment occurred during the course of Experiment 1, the cows were fed the same amount of mixture daily. This avoided any variation in the level of intake of carotene which might complicate the digestibility determinations. The digestibilities in this experiment were determined by the chromium ratio method as outlined in the previous section. To 31.5 cwt. of the carotene containing diet (Diet 2), 2100 g. of Cr_2O_3 were added so that the animals consumed daily about 5 g. chromium (Cr) per head. No marker was added to the carotene-

free diet (Diet 1). A representative sample of faeces was collected every second day for the determination of carotene, dry matter and chromium. Milk samples were also collected every second day, and their carotene and vitamin A were partitioned and estimated quantitatively.

The experiment with goats (Exp.2) was divided into three periods. A diet consisting of 9 parts by weight of oats, 6 parts by weight of decorticated earth-nut cake and 6 parts by weight of grass meal, was fed throughout the experiment. Each animal received 3 lb. of concentrate mixture and 1 lb. of oat glumes daily. The oat glumes were used because they were readily weighed, contained no carotene and required no chopping. The experiment consisted of a preliminary period (Period 1) of 20 days. The animals were placed in specially built individual metabolism cages with attach-ments, designed by Dr K.L. Blaxter for the quantitative separation and collection of faeces in goat experiments which he carried out at Weybridge. Every second morning at 10 a.m. the total collection of the faeces for the preceding 48 hours was accurately weighed and a well mixed sample taken for immediate analysis of carotene. Collections were made wvery 2-days during the last ten days of this period (Period 1). At the termination of the period, the animals were taken out of the cages but were kept indoors on the same diet.

The treatment period (Period 2) lasted two weeks, during which time the animals were again kept in the individual metabolism cages. They were divided into

four pairs of which the first pair received 10 mg. thyroxine/head/day, the second pair received 20 mg. thiouracil/head/day and the third pair 10 mg. thyroxine and 1 mg. stilboestrol/head/day. The drugs were all administered by subcutaneous injection. Stilboestrol was injected in olive oil solution (Malpress & Owen, 1948). The fourth pair of animals were kept as untreated controls. It was originally intended to continue this period for 20 days but due to loss of one animal from each of the first and third pairs the treatments were discontinued at the end of the fourteenth day. At the termination of the period, the animals were again removed while the cages were cleaned. They were then put back in the cages for a further 20 days which acted as the post treatment period (Period 3). Throughout Period 2 and 3 composite faeces samples were collected and analysed for carotene every two days. The animals were weighed initially and at the end of each period. The digestibilities were calculated for each animal from the data obtained every two days.

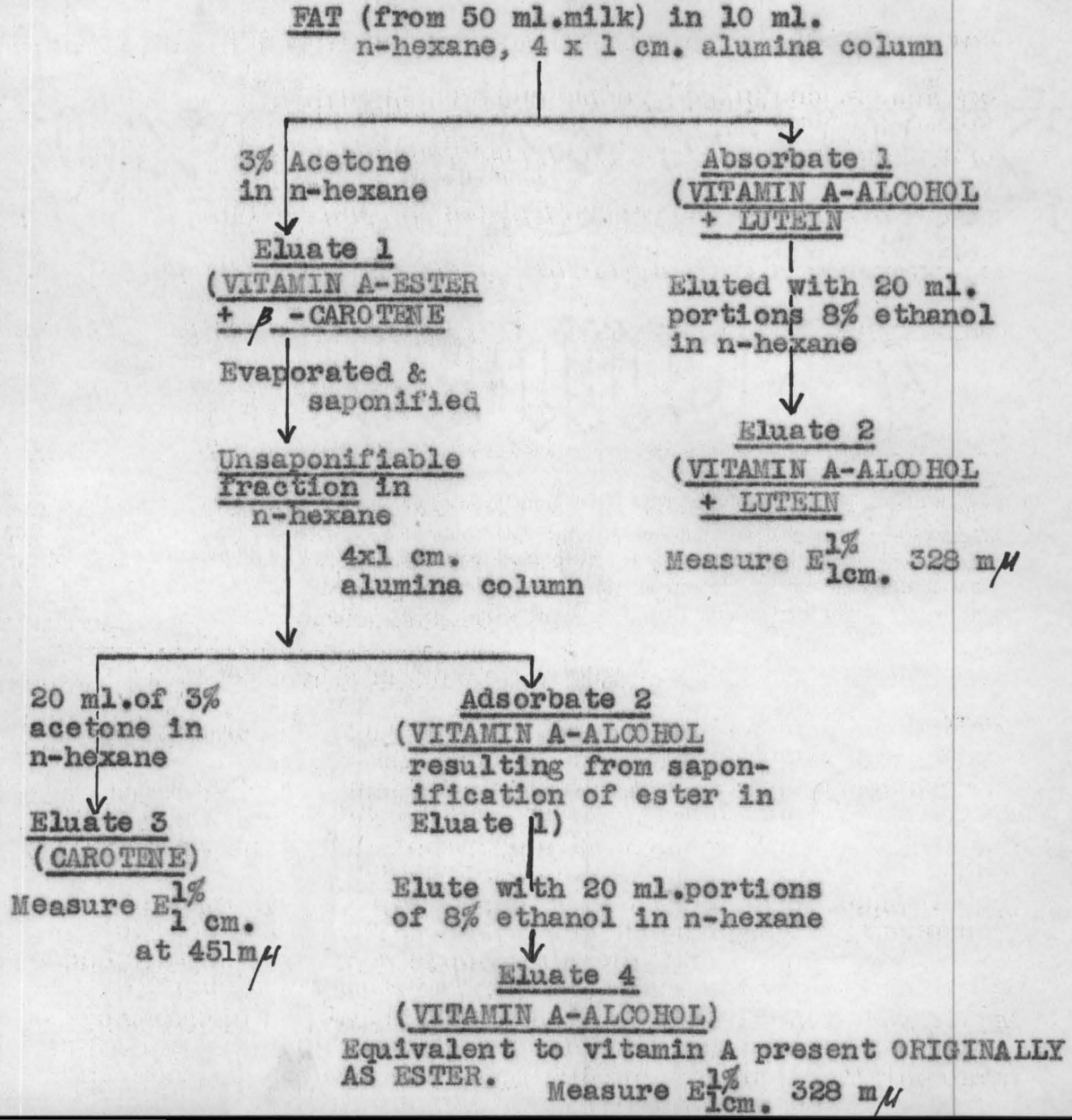
Samples of the morning and evening milk from individual animals were pooled every second day in proportion to the yields, and the mixed sample analysed for fat and vitamin A.

Methods of analysis

The methods for estimating chromium and carotene in faeces have been described in detail in the previous section of this Chapter. Carotene and differ-

ent forms of vitamin A in milk were determined in the Unicam Spectrophotometer after chromatographic separation of the extracted fat. The method is described in detail in Chapter IV which deals with human milk. The milk fat of cows and goats, unlike human milk fat, does not contain lycopene. A brief outline of the essential chemical steps followed in these experiments for the separation of carotene and vitamin A is shown below.

Separation of carotene, lutein, vitamin A-alcohol and vitamin A-ester by chromatography on alumina



Determination of total vitamin A

In the experiments on human milk the total vitamin A was determined by adding the values found for vitamin A ester and vitamin A alcohol. In the present experiments, however, the total vitamin A was determined separately. This was possible because of the large quantities of milk which were available for analytical purposes. For the determination of total vitamin A, the fat was extracted from 50 ml. milk and saponified. The unsaponifiable residue was dissolved in 5 ml. n-hexane and chromatographed on alumina. The carotene was eluted with 3% acetone in n-hexane. Vitamin A alcohol which remained adsorbed in the column after the elution of the carotene was eluted separately with 8% ethanol in n-hexane. The eluate was evaporated under reduced pressure and the residue dissolved in 5 ml. n-hexane for measurement of extinction at 328 mμ.

In order to calculate the total vitamin A activity, 0.6 μg. β-carotene was assumed to be equivalent to 1 i.u. vitamin A. The validity of this conversion factor is still a matter of conjecture but in view of the generally accepted international standard, its use in the present work was justified. It will be seen later that the results of the present studies suggest that the conversion factor is not correct (p.153).

RESULTS

General

The accuracy of the chromatographic separation of vitamin A into free and esterified forms was tested by adding different forms of vitamin A to the fat extracted from the milk of cows and goats. The results are shown in Table 35. Initially the average figure for the cows' milk was only 5 i.u. vitamin A alcohol per 100 ml., and there was no measurable quantity of free vitamin in the goats' milk. It will also be seen that the recovery of added vitamin A was between 90-98% when the total quantity in the final solution for optical measurement was 20 i.u. or more. When smaller amounts were present the recovery was only about 80%. Recovery was never complete because the loss of some vitamin A during saponification and chromatography could not be avoided even under rigorous conditions. Table 35 shows that the loss during the determination can be up to 20% when dealing with less than 20 i.u. vitamin A.

The initial vitamin A activity present in the milk of the experimental cows is shown in Table 36 and of the experimental goats in Table 37. The determinations were made during the week before the first experimental period began and represent typical figures under winter feeding conditions. All the six cows gave comparable milk yields (Table 36). The coefficient of variation of the initial yield was only $\pm$ 4.3%. The coefficient of variation in the carotene concentration was higher than that of vitamin A (16.0 compared with

10.2). The highest vitamin A value (108 i.u./100 ml.) corresponded with the highest fat content of 4.7%. The further partition of vitamin A to alcohol and ester showed that the ester was a constant proportion of the total. The mean value was found to be 92.5 ± 1.9%. The mean vitamin A alcohol was 5.8% of the total vitamin. The individual values ranged between 4.6 - 7.5 i.u./100 ml. milk and thus gave a coefficient of variation ± 19.2%. It should be noted that the values for the alcohol were direct measurements and not derived by difference, a fact which explains the lack of equality between the two foregoing coefficients of variation. Of the total vitamin A activity (vitamin A + carotene), carotene contributed on the average 24.8% (assuming that 0.6 μg. β-carotene = 1 i.u. vitamin A). The proportion was practically constant for all the animals, the range being 22.1 - 28.0 (c.v. ± 3.4%).

Table 37 shows the concentration of carotene in goats' milk. The values ranged from 110 - 174 i.u./100 ml. milk. The coefficient of variation (± 15.4%) was higher than that for the carotene content of the cow's milk. Of the vitamin A 98% was attributable to esters and there was no measurable amount of the alcoholic form. The coefficient of variation of the mean percentage of vitamin A as ester was only ± 0.5%. A comparison of Table 36 and 37 shows that there was more vitamin A in the goat's milk than in the cow's milk and that the fat of goats' milk was richer in vitamin A than that of the cows' milk. The lowest

vitamin A value for the goats was higher than the mean value of the vitamin A plus carotene (27.8 i.u. total potency/g. butter fat) for the cows. The mean value per gram of goat butter fat was 39 i.u. and it was present exclusively as preformed vitamin A. Traces of carotene were present from time to time in goat's milk but they were so small that they could not readily be measured.

Table 34. The composition of the diets fed to the cows

Components	Diet 1 (Carotene-free)	Diet 2 * (Carotene)	% S.E.	% D.C.P.
Oats	9 parts	9 parts	60	8
Bean meal	6 "	6 "	66	20
Grass meal	-	6 "	60	11
Carotene-free substitute for grass**	6 parts	-	60.5	11.4
Starch equivalent (S.E.) lb./21 lb.	13.0	13.0	-	-
Digestible crude protein (D.C.P.) lb./21 lb.	2.60	2.58	-	-

* Diet 2 contained 2100 g. Cr_2O_3/31.5 cwt.

** Composition of carotene-free substitute for grass meal.

Components	% in the carotene-free substitute for grass meal
Blood meal (63% S.E. and 73% D.C.P.)	15.0
Ground oat straw (20% S.E. and 1% D.C.P.)	42.5
Ground potato starch (100% S.E.)	42.5

Table 35. The recovery of the ester and alcohol forms of vitamin A when added to milk fat

	Nature of milk fat								
	Vitamin A in original milk fat from 100 ml. milk(i.u.)		Vitamin A added (i.u.)	Vitamin A in fortified milk fat (i.u.)					
				Ester			Alcohol		
	Ester	Alcohol		Expected	Found	% added vitamin recovery	Expected	Found	% added vitamin recovery
Cow									
1	71	6	50 (ester)	121	120	98	6	5	-
2	67	4	30 (ester)	97	95	93	4	5	-
3	99	7	10 (alcohol)	99	96	-	17	15	80
4	88	5	20 (alcohol)	88	92	-	25	23	90
5	77	5	30 (alcohol)	77	80	-	35	34	97
6	84	4	50 (alcohol)	84	81	-	54	52	96
Goat									
1	184	-	30 (ester)	214	212	93	-	-	-
2	122	-	50 (ester)	172	170	96	-	-	-
3	106	-	5 (alcohol)	106	105	-	5	4	75
4	132	-	10 (alcohol)	132	129	-	10	8	80
5	115	-	20 (alcohol)	115	118	-	20	18	90
6	155	-	30 (alcohol)	155	159	-	30	29	97
7	165	-	40 (alcohol)	165	161	-	40	38	95
8	141	-	50 (alcohol)	141	138	-	50	47	94

Table 36. The initial vitamin A activity present in the milk of the experimental cows

	Name of cow						Mean	Coefficient variation
	Dora	Tinker	Delilah	Griselda	Jean	Gwynneth		
Milk yield (kg./2 days)	30.2	28.6	28.8	28.9	32.0	29.7	29.7	4.3
Fat in milk (%)	4.1	4.4	3.9	4.2	4.7	3.9	4.2	7.4
Carotene (μg./100 ml. milk)	18.5	12.1	19.5	17.6	19.4	16.5	17.3	16.0
Vitamin A (i.u./100 ml. milk)	79.5	71.4	83.8	89.7	107.9	93.5	87.6	10.2
Proportion of the vitamin A present as ester (%)	89.4	93.7	91.9	93.5	92.5	94.0	92.5	1.9
Proportion of the vitamin A present as alcohol (%)	7.5	4.7	5.9	4.6	6.5	5.4	5.8	19.2
Carotene (μg./g. fat)	4.5	2.8	5.0	4.2	4.1	4.2	4.1	18.2
Carotene (i.u./g. fat)	7.5	4.6	8.4	7.0	6.9	7.1	6.9	18.2
Vitamin A (i.u./g. fat)	19.4	16.2	21.5	21.4	23.0	24.0	20.9	13.3
Total vitamin A activity (i.u./g. fat)	26.9	20.8	29.9	28.4	29.9	31.1	27.8	13.3
Amount of the vitamin A activity due to carotene (%)	28.0	22.1	28.0	24.7	23.1	22.8	24.8	10.6

Table 37. The initial vitamin A activity in the milk of the experimental goats

Goats	Milk yield (ml./2 days)	% Fat	Vitamin A (i.u./100ml. milk)	Vitamin A (i.u./g.fat)	Proportion of the total present as ester (%)
Betty	2670	3.1	173.7	56.0	98.5
Judith	1940	4.1	119.6	29.2	97.9
Anna	4600	4.3	164.8	38.3	99.2
Diana	1780	4.2	150.9	35.9	97.8
Mazy	1330	3.8	128.2	33.7	98.6
Heather	2270	3.0	141.7	47.2	98.1
Bluebell	2730	4.0	110.0	27.5	98.9
Miranda	3760	3.4	150.2	44.2	98.2
Mean	2635	3.7	142.4	39.0	98.4
Coefficient of variation	41.0	13.6	15.4	24.7	0.5

Absorption of carotene by cows

The average carotene contents of the concentrate mixtures fed to cows in different periods are shown in Table 38. There was a gradual loss of carotene with the progress of the experiment. A fresh mixture was made at the beginning of each period using dried grass which was stored in brown paper bags. The carotene concentration ranged from 5.37 to 4.85, 5.30 to 5.03, and 5.20 to 4.78 mg. per 100 g. during Period 2, 4 and 5 respectively. The higher concentrations corresponded to the beginning and the lower ones to the end of each period. The average chromium concentration was also fairly constant in the different periods. The coefficient of variation of Cr were 15.9, 10.2 and 4.2 % in Periods 2, 4 and 5 respectively (Table 38). It will be of interest to note that the mixtures were made in that order and that the efficiency of mixing increased with experience. The ratios of carotene to chromium expressed as mg. carotene per g. chromium, which were used in calculating digestibility, are also shown in Table 38.

The faecal carotene concentration throughout the experiment is shown in Fig. 15 for a control cow, for a cow receiving thyroxine and for a cow receiving thiouracil. The corresponding results for the other three cows are shown in Fig.16. It will be observed from the two figures that the initial concentration of carotene varied between 2.5 mg. and 3.8 mg./100 g. dry faeces. When the carotene-free diet was given the concentration dropped rapidly and attained a threshold

value of 100 μg./100 g. within a week. When the cows were again given the carotene diet the faecal concentration rose sharply and became constant in 6 to 8 days. The animals were all eating equal amounts of carotene and the concentrations of carotene (5.3 - 5.7 mg./100 g.) in the faeces did not vary much from cow to cow. In Period 4, during which thyroxine or thiouracil was given the treated animals showed rates of increase in faecal carotene which were significantly different from those of the control animals. Thus it will be observed from Fig.15 that the concentrations for the control animal, Dora, changed in the same way as in Period 2, the carotene concentration in the faeces increasing during the first few days and attaining a fairly constant value in about 8 days, whereas in the thyroxine treated animal, Delilah, the maximum attained on the eighth day was only 3.2 mg./100 g. as compared with 4.9 mg. at the corresponding stage in the control animal. Again it will be noticed that in the control animal, the concentration remained relatively steady after the attainment of the maximum, whereas in the thyroxine treated animal attainment of the maximum was followed by a sharp drop during the next four days when the concentration became only 1.8 mg./100g. This in turn was followed by a further drop during the next 8 days to a concentration of 1.5 mg./100g. towards the end of the treatment period. From Fig.15, it can be seen that for the cow receiving thiouracil the value increased even after the eighth day, attaining a figure of 7.1 mg./100 g. on the twelfth day of treatment and

remaining at about 6 - 6.5 mg./100 g. till the end of the period. The rates of reappearance of carotene in the faeces have been expressed in numerical form in Table 39 which records the slopes (expressed as μg. increase of faecal carotene per day) of straight lines fitted by the method of least squares to the faecal excretions of Periods 2 and 4. During Period 2 the rates of reappearance of faecal carotene (Table 39) were comparable for all six cows, the average being 277 μg./day with a coefficient of variation of ± 2.7%. During treatment (Period 4), these rates of reappearance of faecal carotene were reduced by thyroxine and increased by thiouracil.

The average faecal carotene concentrations per 100 g. dry faeces in Periods 2, 4 and 5 when carotene was fed, are shown in Table 40 together with carotene intakes and the apparent digestibilities determined by the chromium method. It will be observed that the mean digestibilities were higher during thyroxine treatment and lower during thiouracil treatment. The digestibility of carotene in the thyroxine cows during the treatment period was 34% above the pre-treatment figure, while in the thiouracil cows the digestibility was decreased by 12%, the values for the control cows showing relatively little change.

The treatment period was divided into five sub-periods of 4-days duration for determining digestibilities. The individual values for each sub-period are recorded in Table 41 for all the six cows. It will be observed

that a value as high as 84.5% was obtained during thyroxine treatment and one as low as 32.9% during thiouracil treatment. The analysis of variance, also shown in the table, indicates that there was a significant degree of variation between the mean digestibilities for the different animals. This was evidently due to the treatment since no such significant difference between cows was observed during the pre-treatment period.

Table 38. The carotene and chromium contents of the food of the cows during the periods when the carotene diet was fed

	Period 2	Period 4	Period 5
Carotene (mg./ 100 g. diet)	5.20	5.16	5.13
Coefficient of variation (%)	5.1	2.2	4.6
Chromium (mg./ 100 g. diet)	69.2	69.1	71.6
Coefficient of variation (%)	15.9	10.2	4.2
mg.carotene/ g.Cr.	75.1	74.7	71.7

Fig 15

The Effects of Diets and Drugs on the Faecal Excretion of Carotene

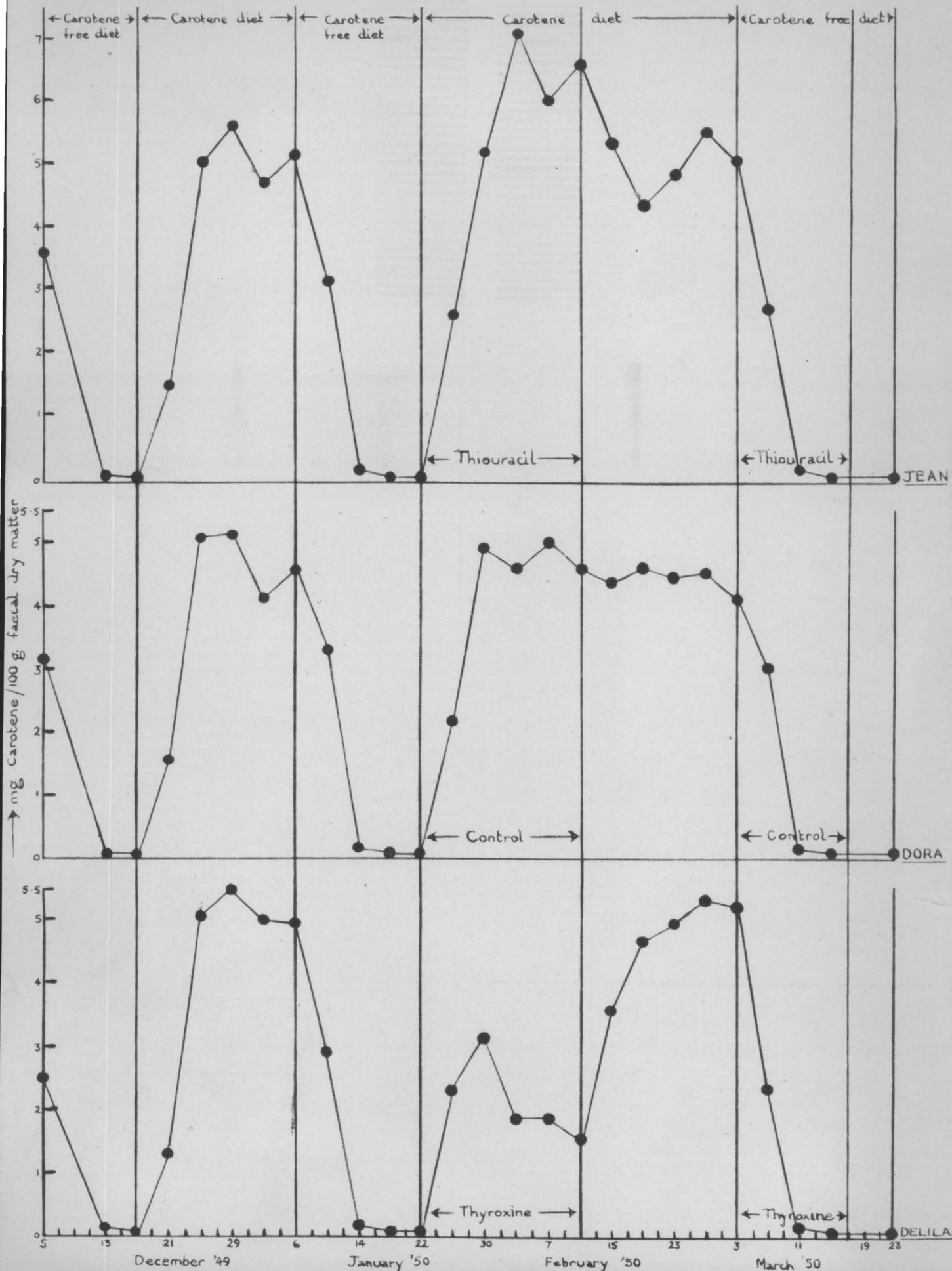


Fig. 16

The Effects of Diets and Drugs on the Faecal Excretion of Carotene

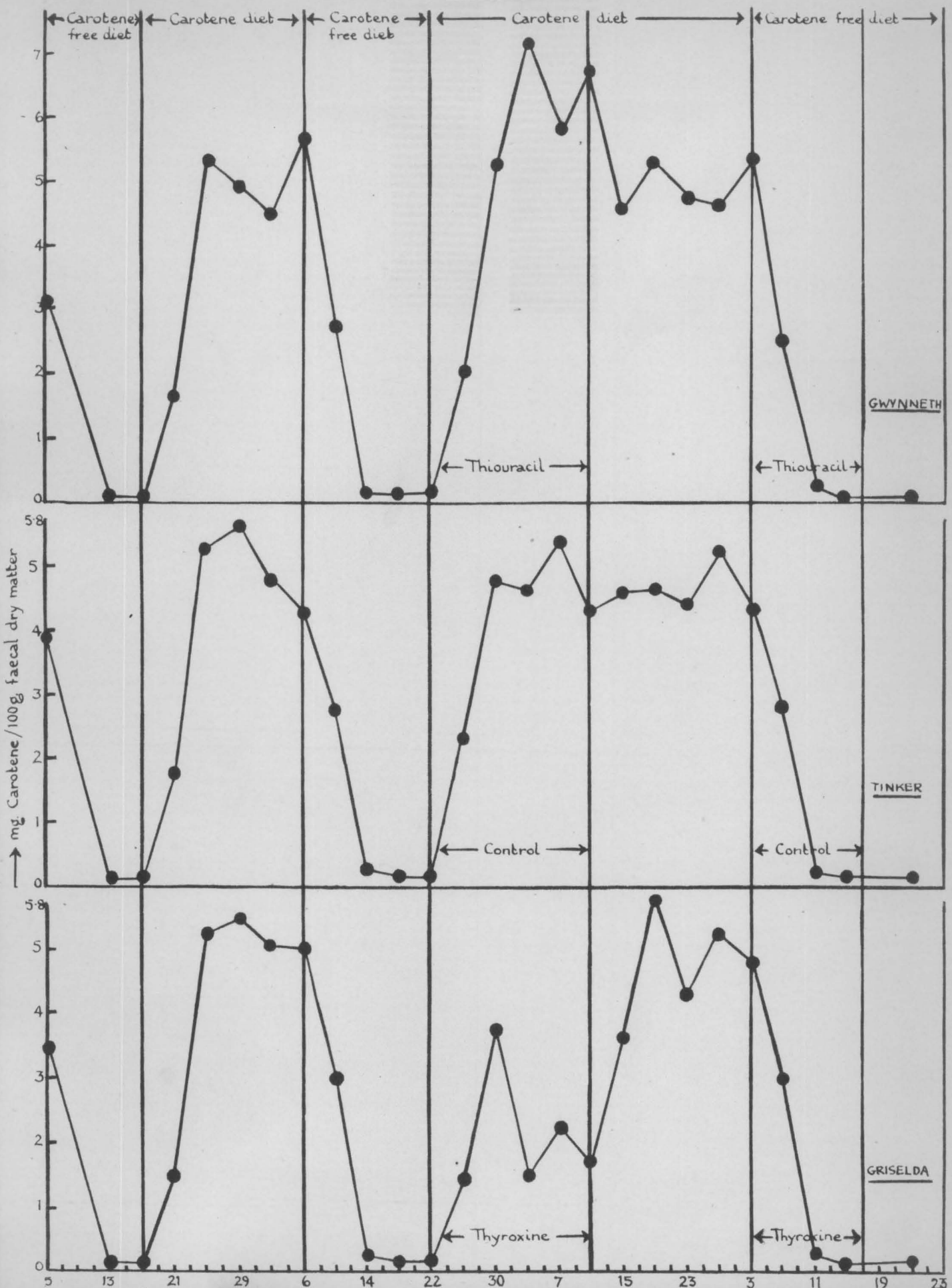


Table 39. The effect of thyroxine and thiouracil on the rate of increase in the concentration of carotene in the faeces of cows, when a carotene diet replaced a diet free from carotene

Cows	Rate of increase in the concentration of carotene in the faeces (µg. carotene/100 g. faeces/day)		Treatment (per day) in Period 4
	In the absence of hormone treatment (Period 2)	During treatment (Period 4)	
Dora	266	247	None
Tinker	286	271	None
Delilah	272	158	10 mg. thyroxine
Griselda	277	188	10 mg. thyroxine
Jean	279	353	20 mg. thiouracil
Gwynneth	284	360	20 mg. thiouracil

Table 40. The effect of thyroxine and thiouracil on the apparent digestibility of carotene in cows

Cows	Period	Carotene intake (mg./2 days)	Faecal Carotene (mg./100g.)	Faecal Chromium (mg./100g.)	mg.carotene/g. Chromium	Carotene excreted (mg.)	Apparent digestibility (%)
Dora	2	977	4.49	145	30.9	402	58.9
	4 (control)	978	4.25	135	31.6	414	57.7
	5	946	4.28	133	32.2	426	55.0
Tinker	2	948	4.58	128	35.9	453	52.2
	4 (control	947	4.29	134	32.1	407	57.0
	5	968	4.57	145	31.5	425	56.0
Delilah	2	973	4.71	133	35.6	460	52.7
	4 (thyroxine)	957	2.49	110	22.6	289	69.8
	5	957	4.65	154	30.2	404	57.8
Griselda	2	963	4.79	136	35.1	451	53.2
	4 (thyroxine)	980	2.45	122	20.0	263	73.2
	5	950	4.79	147	32.6	432	54.5
Jean	2	968	4.71	145	32.5	419	56.7
	4 (thiouracil)	983	5.50	151	36.5	480	51.1
	5	929	4.85	146	33.2	431	53.6
Gwynneth	2	929	4.60	137	33.6	415	55.3
	4 (thiouracil)	973	5.35	137	39.0	508	47.8
	5	973	4.78	152	31.4	426	56.2

Table 41. The digestibility coefficient of carotene in cows determined every 4 days during a period of hormone treatment

Sub-periods	Cows					
	Dora (control)	Tinker (control)	Delilah (thyroxine)	Griselda (thyroxine)	Jean (thiouracil)	Gwynneth (thiouracil)
1	64.5	68.1	69.3	64.6	71.3	64.9
2	52.7	56.7	55.4	69.9	49.2	40.7
3	56.6	52.4	75.3	75.6	43.6	48.5
4	48.8	48.2	68.8	71.5	40.1	32.9
5	65.8	59.3	80.2	84.5	51.5	51.8
Mean	57.7	57.0	69.8	73.2	51.1	47.8

Analysis of variance of the above data

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (ε^{2Z})
Total	29	4742.40	-	-
Subperiods	4	1107.72	276.930	5.151 **
Animals	5	2559.38	511.876	9.521 ***
Error	20	1075.30	53.765	-

Absorption of carotene by goats

The average concentration of carotene in the food and faeces at different periods is given in Table 42. It will be seen that the concentration of carotene in the food was highest in Period 1 (3.14 mg./100 g.) and lowest in Period 3 (2.71 mg./100 g.). The concentrate mixture was made in one batch and used throughout the experiment, but as will be observed from Fig.17 the carotene concentration progressively decreased so that 24% of the original potency was lost in forty days.

The digestibility of carotene as determined by the direct method and the average daily intake and faecal excretion are shown in Table 43. It will be seen from Tables 42 and 43 that both the concentration and daily output of carotene in the faeces were increased when the animals were treated with thiouracil. In the goat, Anna, the digestibility was decreased from 68% in the pre-treatment period to 54% in the treatment period and in the goat, Diana, the corresponding figures were 62 and 48%. On cessation of treatment the digestibilities in both goats returned towards the original values. In the two thyroxine treated goats Mazy and Heather, the digestibilities were increased due to treatment. Thus during thyroxine treatment the value for both goats was 74% as compared with 63% and 66% in the pre-treatment period, while in the control goats the digestibilities remained fairly constant throughout the experiment. The administration of stilboestrol along with thyroxine in the two goats, Bluebell and Miranda, did not appear

to affect the action of thyroxine in bringing about an increase in the digestibility of carotene.

Table 42. The carotene content in the food and faeces of the goats in the three periods of the experiment

Food carotene (mg./100g)		Period 1	Period 2	Period 3
		3.14	2.97	2.71
Goat	Treatment per day in Period 2	Faecal carotene (mg./100g. dry faeces)		
Betty	None	2.54	2.35	2.09
Judith	None	2.18	2.05	1.39
Anna	20 mg. thiouracil	2.24	3.42	2.49
Diana	20 mg. thiouracil	2.97	3.37	2.20
Mazy	10 mg. thyroxine	2.05	0.99	2.52
Heather	10 mg. thyroxine	1.97	1.31	-
Bluebell	10 mg. thyroxine + 1 mg. stilboestrol	2.31	1.48	2.50
Miranda	10 mg. thyroxine + 1 mg. stilboestrol	2.71	0.31	-

Fig. 17 Loss, on storage, of carotene from the concentrate mixture fed to goats

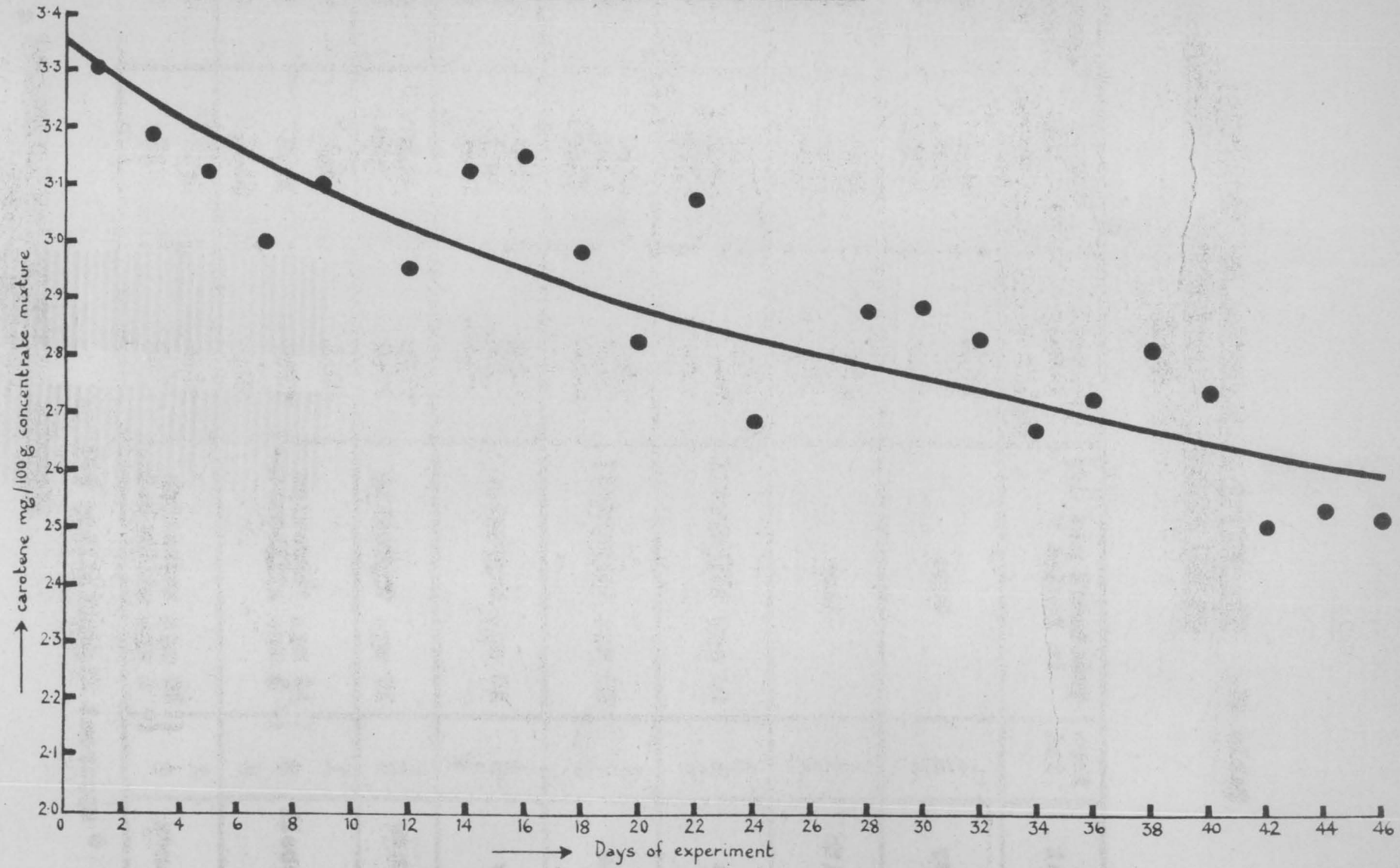


Table 43. The effect of thyroxine and thiouracil on the apparent digestibility of carotene by goats

Goat	Period	Treatment per day in Period 2	Carotene intake (mg./2 days)	Faecal carotene (mg./2days)	Apparent * digestibility (%)
Betty	1	None	83.3	30.8	63.0
	2		74.1	27.4	62.3
	3		59.6	20.9	64.5
Judith	1	None	56.1	21.1	62.9
	2		49.0	18.9	60.7
	3		27.1	10.2	61.3
Anna	1	20 mg. thiouracil	85.5	26.8	68.4
	2		79.6	36.8	53.8
	3		73.7	27.9	62.1
Diana	1	20 mg. thiouracil	82.7	32.1	61.6
	2		65.5	34.8	48.0
	3		55.6	19.6	61.3
Mazy	1	10 mg. thyroxine	72.9	26.8	62.9
	2		41.8	10.3	74.1
	3		65.5	27.9	57.7
Heather	1	10 mg. thyroxine	69.9	23.8	66.0
	2		23.9	6.2	73.9
Bluebell	1		85.1	32.2	62.2
	2	(10 mg. thyroxine (+ 1 mg. stilboestrol	52.0	15.4	71.6
	3		66.3	28.0	60.4
Miranda	1		68.3	24.5	62.2
	2	(10 mg. thyroxine (+ 1 mg. stilboestrol	7.0	1.5	77.1

* apparent digestibility (%) =

$$\frac{\text{carotene intake - faecal carotene}}{\text{carotene intake}} \times 100$$

When these experiments were envisaged, the present writer had no knowledge regarding the safe dose of thyroxine for a goat. It was known, however, from the experiments by Owen (1948a) that it would be unwise to exceed a dose of 10 mg. per day with an Ayrshire cow. Meites & Turner (1948) injected 10 mg. of thyroxine daily to their experimental goats and did not report any ill-effects, but in spite of this it was thought that this dose might prove to be too high for a goat. In the absence of any other report and in view of the fact that the basal metabolism of goats is higher than that of cows (Brody, 1945), it was decided to use the dose of 10 mg. thyroxine per goat per day. The four goats which received thyroxine or thyroxine plus stilboestrol developed signs of severe hyperthyroidism and started to refuse food, thus reducing their carotene intake.

The increased digestibilities in the thyroxine treated goats are easily misinterpretable because of complications arising from the drastic fall in the intake of carotene in these goats during the injection period. During the treatment period the goat Heather consumed 24 mg. carotene per 2 days, while Miranda consumed only 7 mg. per 2-days. However, it may be deduced from the results of the two untreated goats (the intakes of which were decreased during successive periods) that the digestibilities were not improved when the intakes were lowered. Thus in the untreated goat, Betty, the carotene intake per 2 days was

decreased from 83 mg. to 60 mg. but the digestibilities were 63 and 65% respectively, while in the other untreated goat, Judith, which ate 56 mg. carotene per 2 days initially and only 27 mg. per 2 days at the end of the experiment, the digestibilities were 63 and 61%. This behaviour of the control goats makes it reasonably certain that the changes in the digestibilities in the injected goats were due to the hormonal treatments and not to variations in intake. In order to segregate the effects of decreasing intakes from those of hormonal treatments, carotene intakes and excretions for individual 2-day subperiods were analysed by the method of co-variance (Snedecor, 1946) with the results shown in Tables 44, 45 and 46. Table 44 shows that when the results in Period 1 were adjusted to a constant intake the variation in the faecal excretion of carotene from goat to goat was not significant. An analysis by the same statistical method of the data from Period 2 (Tables 45 and 46) shows that when the results of the faecal excretions were adjusted to a constant intake, the thiouracil goats excreted significantly more carotene than the controls while the controls excreted more than the thyroxine goats.

Table 44. The intake and excretion of carotene in individual subperiods of the first control period (Period 1) and the analysis of covariance (mg./2 days)

x = carotene intake
y = carotene excreted

Goats	Betty		Judith		Mazy		Heather		Anna		Diana		Bluebell		Miranda	
Sub-period	x	y	x	y	x	y	x	y	x	y	x	y	x	y	x	y
1	87.2	30.2	75.6	28.7	75.0	26.5	90.1	28.3	90.1	22.4	88.2	27.0	90.1	34.4	90.1	31.1
2	86.5	31.7	86.5	36.0	86.5	36.4	84.5	29.4	86.5	26.8	79.7	31.4	86.5	36.6	85.2	30.1
3	79.6	32.3	24.3	9.6	39.8	15.6	79.6	27.2	84.9	21.8	81.3	34.9	84.0	21.5	77.8	26.1
4	80.8	29.9	51.1	16.8	80.8	28.0	68.9	25.8	81.6	39.8	80.8	35.0	81.6	30.3	68.0	25.2
5	82.6	29.9	43.2	14.5	82.6	27.8	26.4	8.5	84.3	23.1	83.5	32.3	83.5	38.2	20.6	10.0

The analysis of covariance of the above data and test of significance of the adjusted mean excretions

Source of variation	Degrees of freedom	Sum of squares and products			Errors of estimate			
		Sx^2	Sy^2	Sxy	Sum of squares	Degrees of freedom	Mean square	F
Total	39	13706.13	2326.61	4654.72	745.83	38	-	
Animals	7	3849.12	589.78	1321.93				
Error	32	9857.01	1736.83	3332.79	609.97	31	19.676	
								0.986 (N.S.)
		For test of significance of adjusted means			135.86	7	19.409	

Table 45. The intake and excretion of carotene (mg./2 days) in individual subperiods of Period 2 when two of the cows received thyroxine and another two thiouracil. The analysis of covariance is also given

x = carotene intake
y = carotene excreted

ats	Betty (control)		Judith (control)		Mazy (thyroxine)		Heather (thyroxine)		Anna (thiouracil)		Diana (thiouracil)	
b-riod	x	y	x	y	x	y	x	y	x	y	x	y
	78.6	30.1	66.0	29.0	80.3	24.1	16.7	4.6	80.3	31.6	77.7	39.9
	84.3	29.9	75.4	30.3	62.1	11.5	24.8	7.7	84.3	45.4	83.4	46.4
	83.9	28.4	64.3	23.9	53.6	10.3	15.2	3.5	83.9	39.0	82.1	48.7
	78.3	23.1	62.3	18.1	38.7	9.6	21.9	6.1	80.8	29.5	80.0	30.7
	60.9	24.6	26.5	12.0	13.2	4.6	35.3	8.6	73.8	37.5	73.0	44.8
	76.8	26.5	26.5	10.7	19.6	4.9	29.7	7.0	81.2	39.5	47.1	26.8
	55.7	28.8	21.7	8.5	25.1	7.3	23.9	6.2	73.2	35.0	15.2	6.3

The analysis of covariance of the above data and the test of significance of adjusted mean excretions

urce of ria tion	Degrees of freedom	Sum of squares and products			Errors of estimate			
		Sx^2	Sy^2	Sxy	Sum of squares	Degrees of freedom	Mean square	F
tal	41	27625.47	7950.48	13134.99	1705.23	40		
imals	5	15784.57	5627.75	8829.10				
ror	36	11840.90	2322.73	4305.89	756.91	35	21.626	8.770 ***
		For test of significance of adjusted means			948.32	5	189.664	(P<0.001)

Table 46. The adjusted mean excretion of dietary carotene by goats during the treatment period (Period 2)

($\bar{x}$ = 55.7 mg./2 days) (b (faecal excretion) = 0.3636 per unit intake)

Goat and treatment	Mean intake(x)	Deviation from experimental mean (x)	bx	Mean observed faecal excretion (y)	Adjusted mean faecal excretion ($y - bx$)	% excreted in faeces (adjusted)
Betty (control)	74.1	+ 18.4	+ 6.7	27.4	20.8	37.3
Judith (control)	49.0	- 6.7	- 2.4	18.9	21.3	38.2
Mazy (thyroxine)	41.8	- 13.9	- 5.1	10.3	15.4	27.6
Heather (thyroxine)	23.9	- 31.8	-11.6	6.2	17.8	32.0
Anna (thiouracil)	79.8	+ 23.9	+ 8.7	36.8	28.1	50.4
Diana (thiouracil)	65.5	+ 9.8	+ 3.6	34.8	31.2	56.0

Effect of thyroxine and thiouracil on the carotene and vitamin A contents of cow's milk

The effects on metabolism of allowing the cows to have carotene after having been deprived of it, were accompanied by changes in the contents of carotene and vitamin A in the milk. These changes are recorded in Figs.18a & b to 23a & b and Tables 47 to 50. From these figures and tables the following conclusions were drawn.

(1) Depriving cows of carotene at any rate for short periods had no effect on the yield of milk or fat. Figs. 18 & 19 substantiate this statement for the control cows and Figs. 20 - 23 for the remaining four cows.

(2) When the diet free from carotene was fed, the carotene content and the vitamin A content of the milk rapidly declined. Carotene declined faster than did vitamin A. Carotene was 9.2, 6.5 and 3.7 μg. per 100 ml. milk in successive subjections of the cow Dora (Fig.18) to the carotene-free diet. The other control cow behaved similarly in this respect.

(3) When the diet containing carotene was fed after one not containing carotene, both carotene and vitamin A in the milk showed increases (Figs. 18 & 19). The rate of these increases was accelerated by thyroxine (Figs. 20 & 21) and retarded by thiouracil (Figs. 22 & 23). These effects of thyroxine and thiouracil are also evident from Table 47. These changes in the carotene and vitamin A in the milk were independent of the percentage of fat in the milk for they were still evident

when the results for carotene and vitamin A were expressed per g. of fat (Table 49 and Figs. 20-23).

(4) Thyroxine treatment superimposed on a carotene-free diet had no effect on the rate of decline of the carotene content of the milk, but caused pronounced fluctuations of the vitamin A content. Marked maxima of vitamin A were observed during the second week of the fortnight's treatment with thyroxine (Figs. 20 & 21). These maxima corresponded to maxima of the proportion of vitamin A present in the alcoholic form (Fig. 24). After discontinuance of thyroxine, but continuing the same carotene-free diet, the concentration of vitamin A in the milk decreased rapidly.

(5) Thyroxine greatly increased the yields of both carotene and vitamin A in the milk of the cows ingesting carotene. By contrast, in cows not receiving carotene, thyroxine increased the yield of vitamin A while the yield of carotene decreased (Figs. 20 & 21).

(6) The diminution in the concentration and yield of vitamin A in the milk which resulted when a carotene-free diet was fed was still more marked in cows receiving thiouracil. On cessation of thiouracil but continuing the carotene-free diet the concentration of vitamin A in the milk showed a transient increase (Figs. 22 & 23).

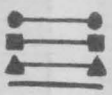
(7) Reference to Fig. 24 and Table 48 shows that the proportion of vitamin A present in the alcoholic form increased whenever a diet free from carotene was eaten.

(8) During period 4 when hormonal treatments were superimposed on a diet containing carotene, the percentages of the vitamin A activity contributed by carotene were 27.7 and 26.6% in the thyroxine cows and 22.1 and 18.2 in the thiouracil cows as compared with 25.8 and 23.4 in the controls (Table 49). These differences may be related to the fact that carotene was more efficiently absorbed during thyroxine treatment. It is probable that in the cow, thyroxine increases the digestibility of carotene partly by stimulating its conversion to vitamin A and partly by increasing its absorption as such.

(9) The recoveries of dietary carotene as vitamin A and carotene in the milk, recorded in Table 50, show that the recoveries in the control cows were much decreased in period 4 as compared with period 2. These decreases were made larger by thiouracil and smaller by thyroxine.

Fig. 18a DORA

Carotene and Vitamin A content in Cow's Milk.



Vitamin A I.U./g. fat
Carotene μg./100 ml. milk
% Fat
Vitamin A I.U./100 ml. milk

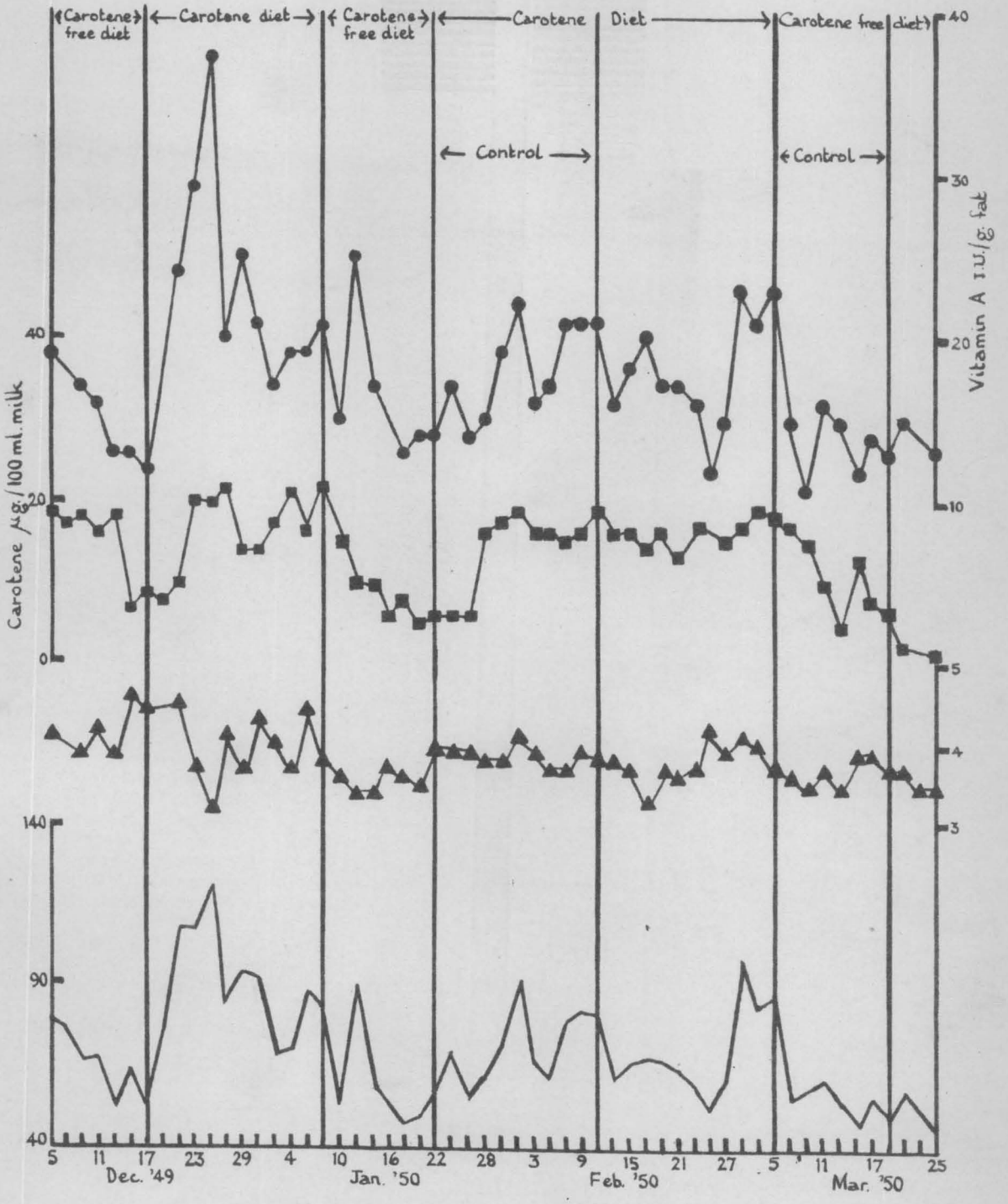


Fig. 18b DORA.

Carotene and Vitamin A content in Cow's Milk

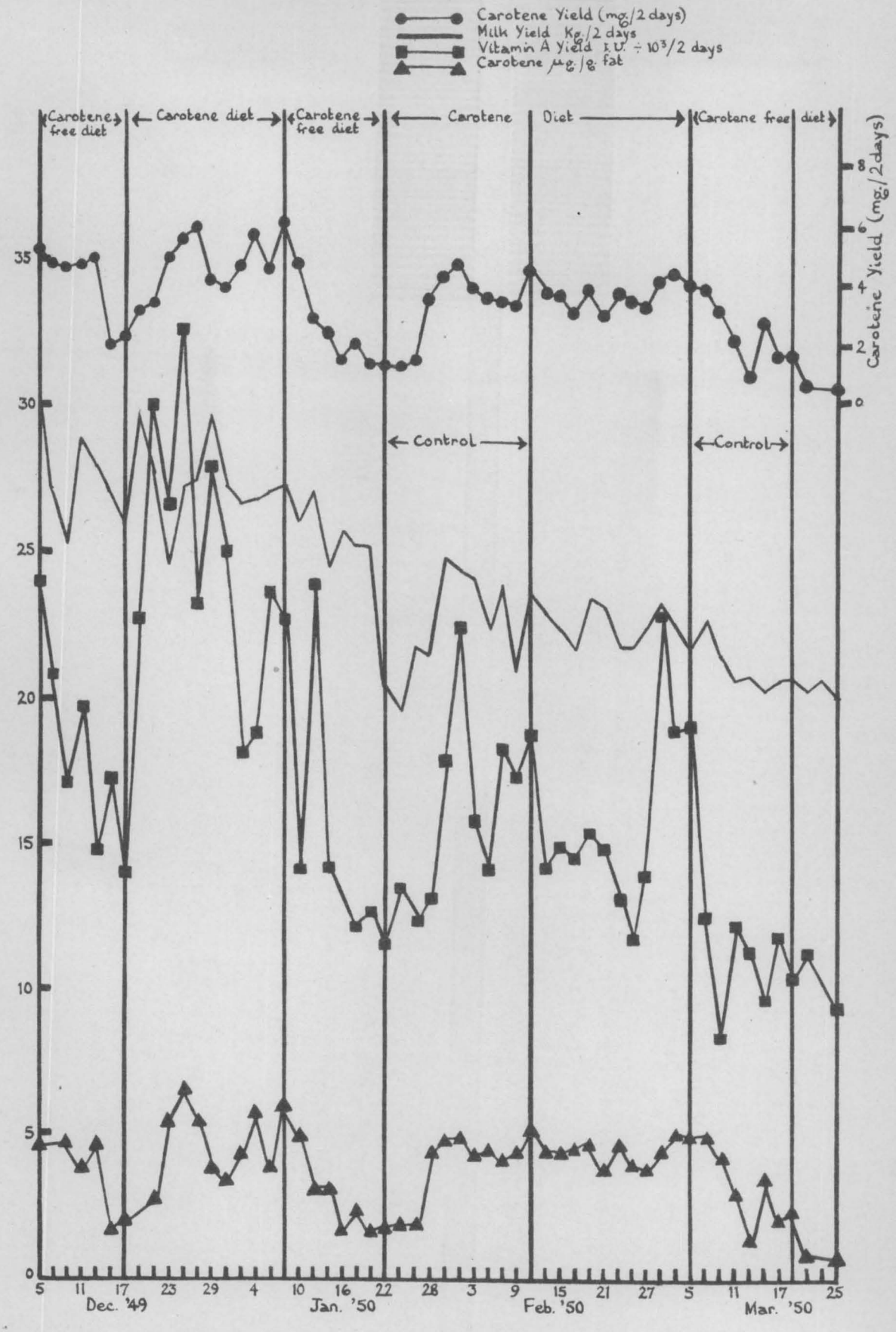


Fig. 19a. TINKER

Carotene and Vitamin A content in Cow's Milk

Vitamin A I.U./g. fat
Carotene µg./100 ml. milk
% Fat
Vitamin A I.U./100 ml. milk

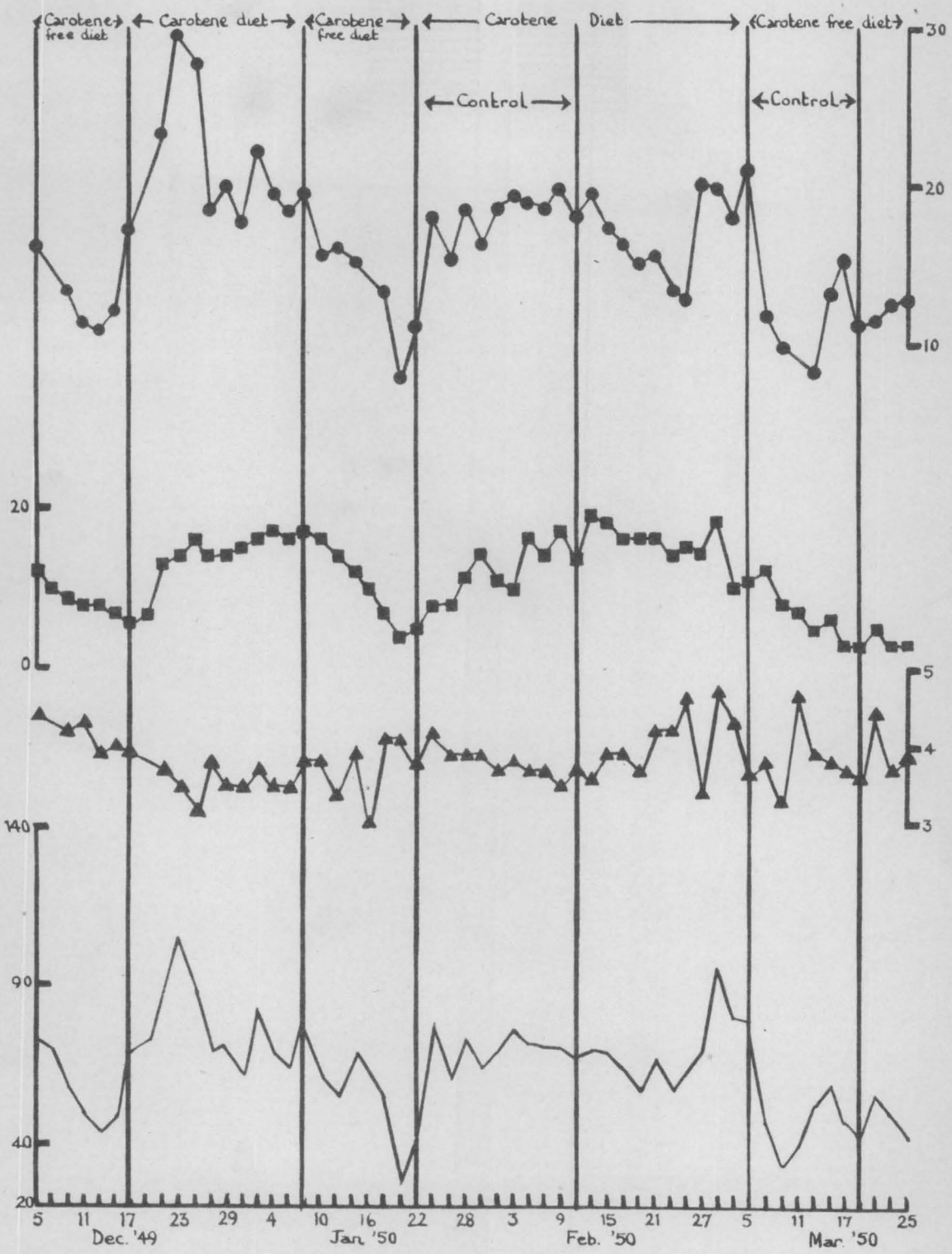
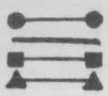


Fig. 19b. TINKER

Carotene and Vitamin A content in Cow's Milk.



Carotene Yield (mg./2 days)
Milk Yield Kg./2 days
Vitamin A Yield I.U. $\div 10^3$/2 days
Carotene μg./g. fat

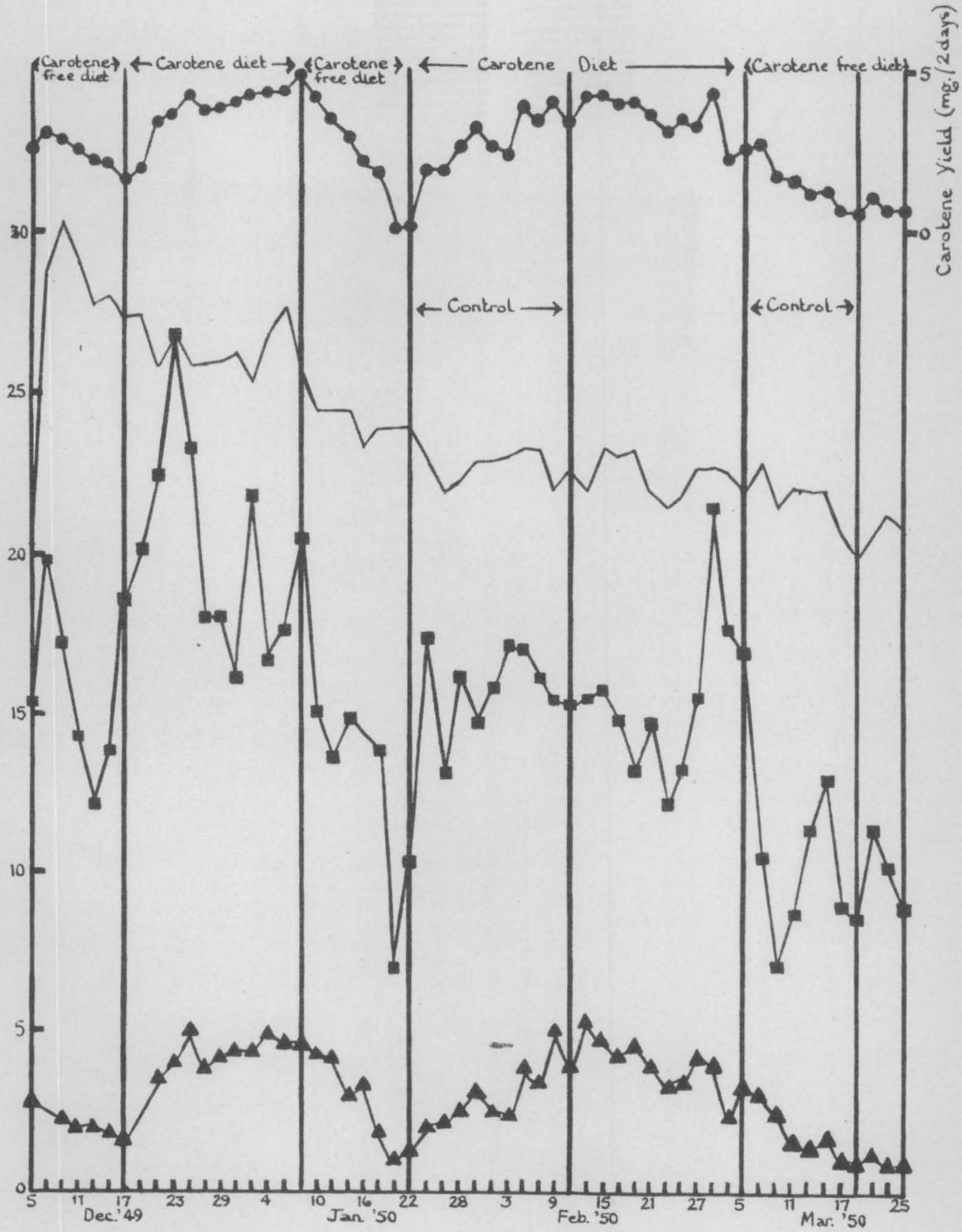


Fig. 20a **DELILAH** Effect of Thyroxine on Carotene and Vitamin A content of Cow's Milk.

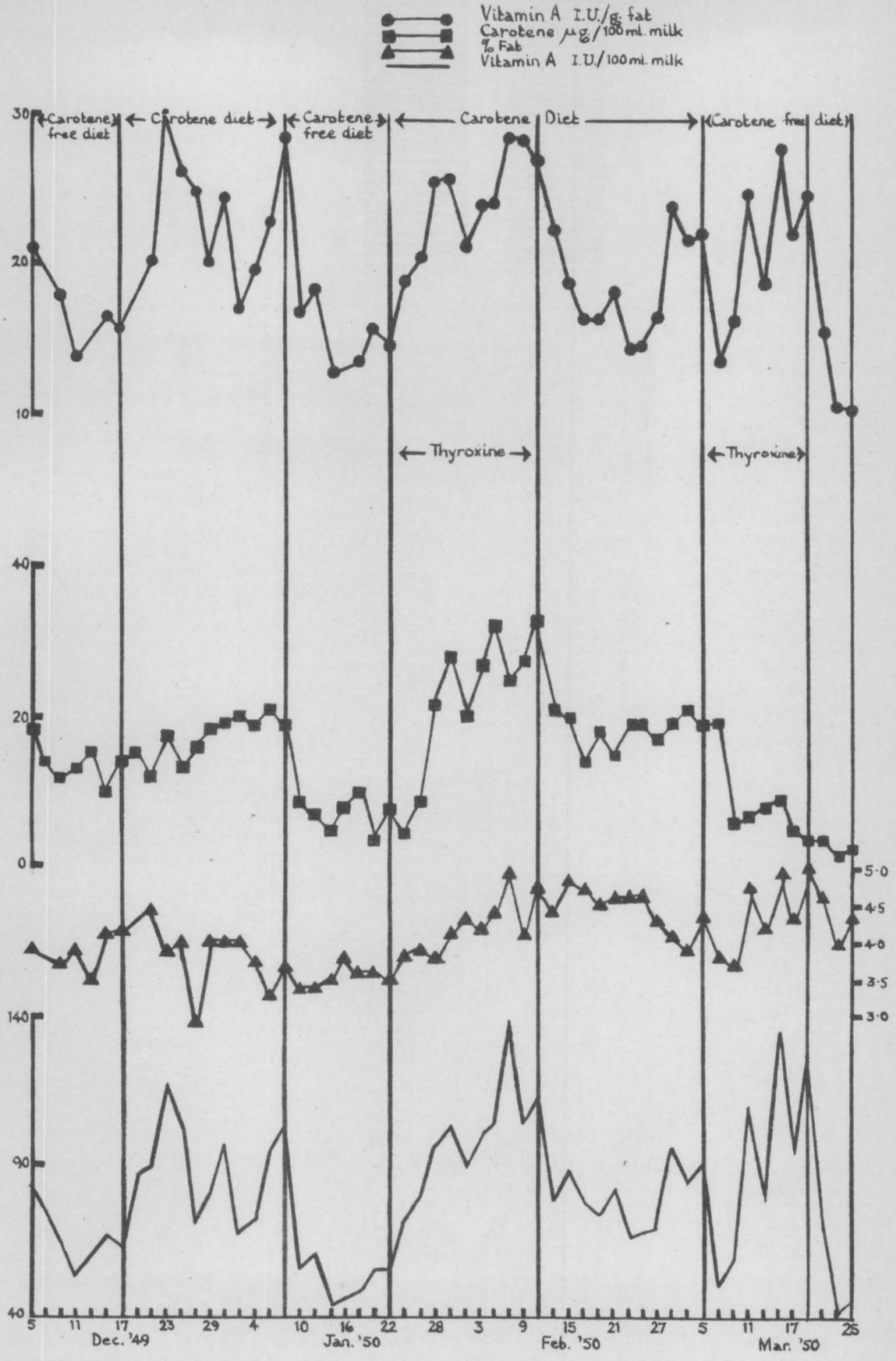


Fig. 20b. DELILAH

Effect of Thyroxine on Carotene and Vitamin A content of Cow's Milk

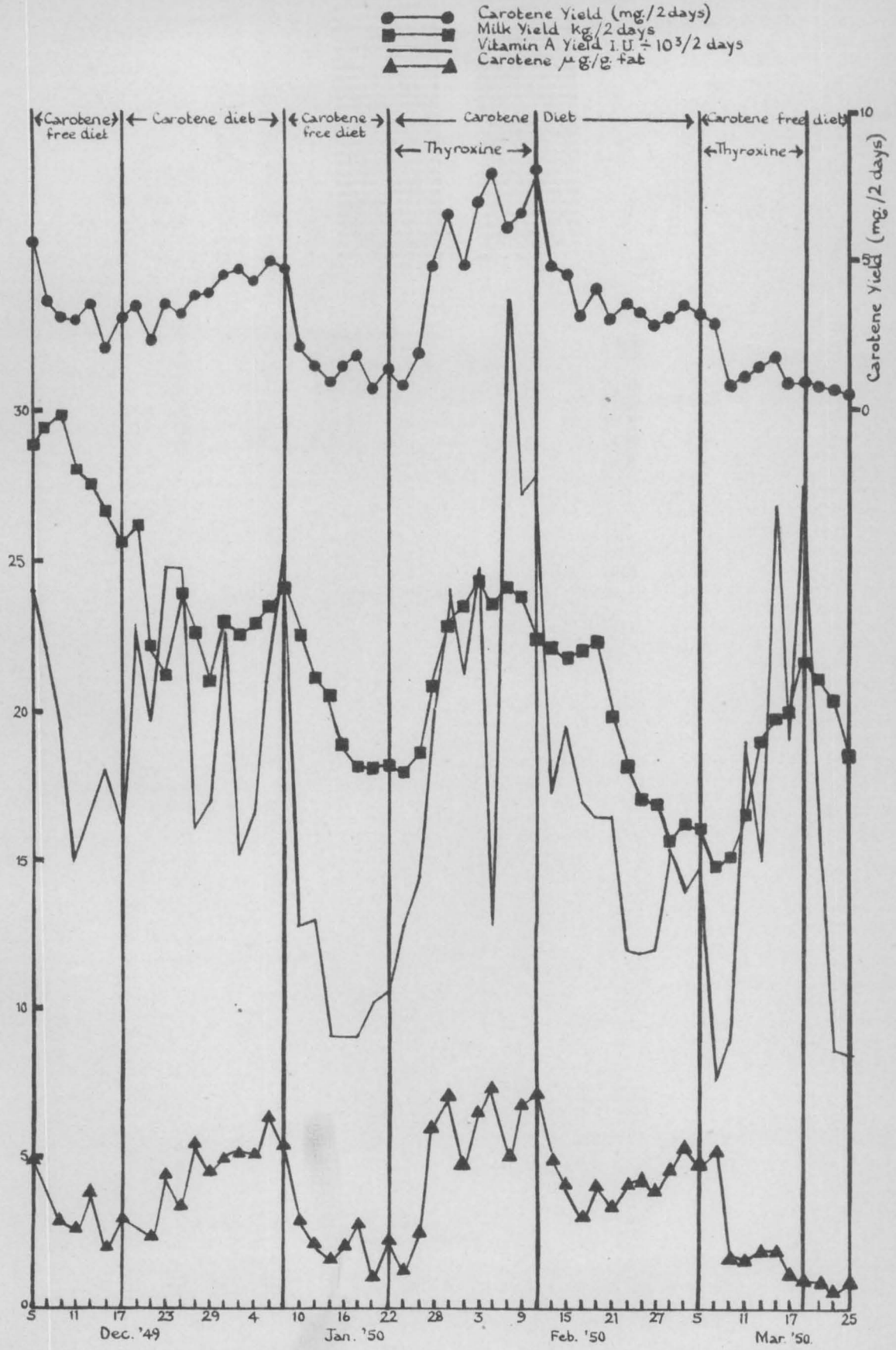


Fig. 21a. GRISELDA

Effect of Thyroxine on Carotene and Vitamin A content of Cow's Milk.

Fig. 21b. GRISELDA

Effect of Thyroxine on Carotene and Vitamin A content of Cow's Milk

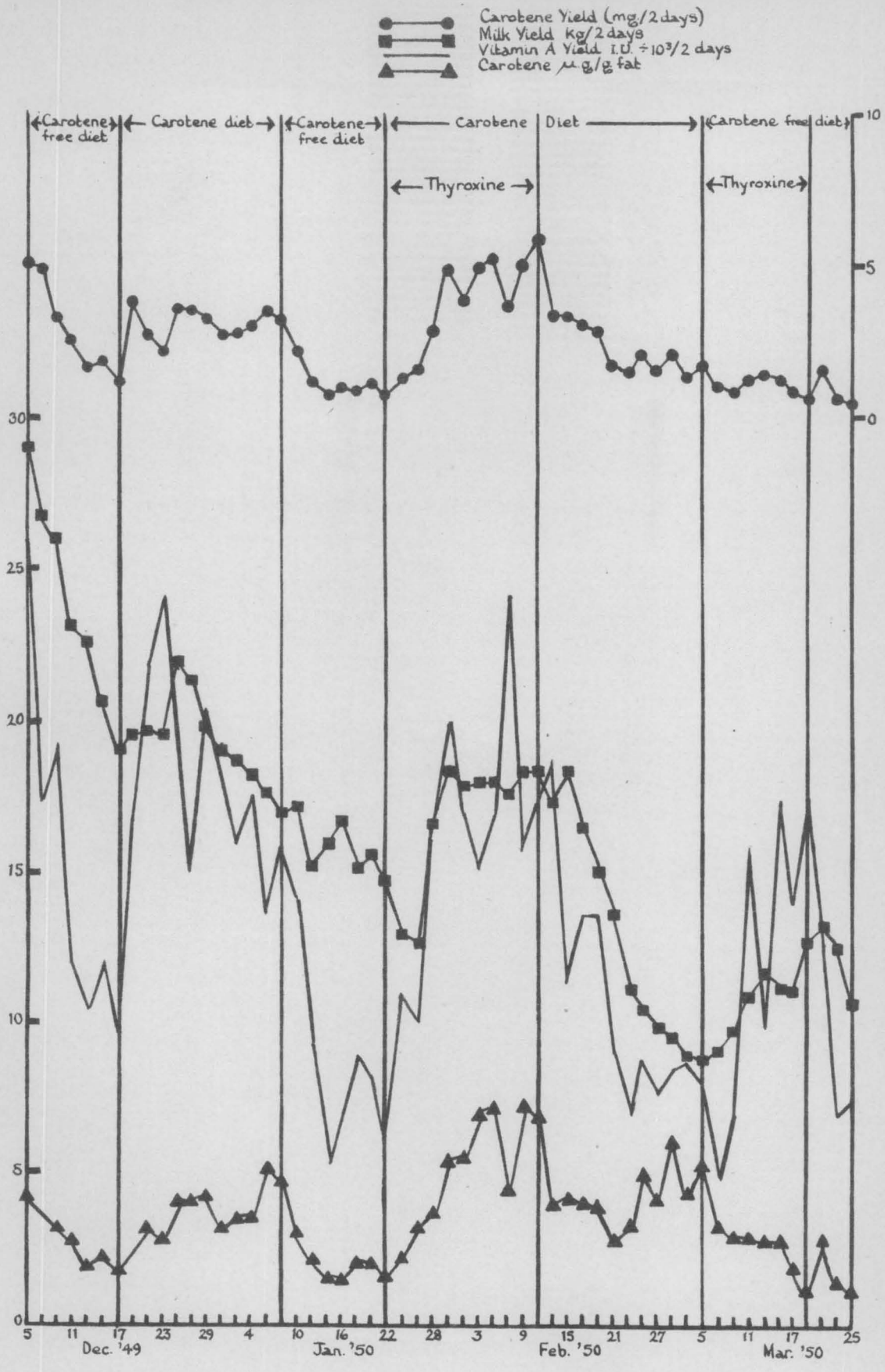
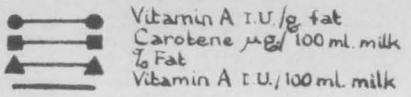


Fig. 22a.

JEAN

Effect of Thiouracil on Carotene and Vitamin A content in Cow's Milk



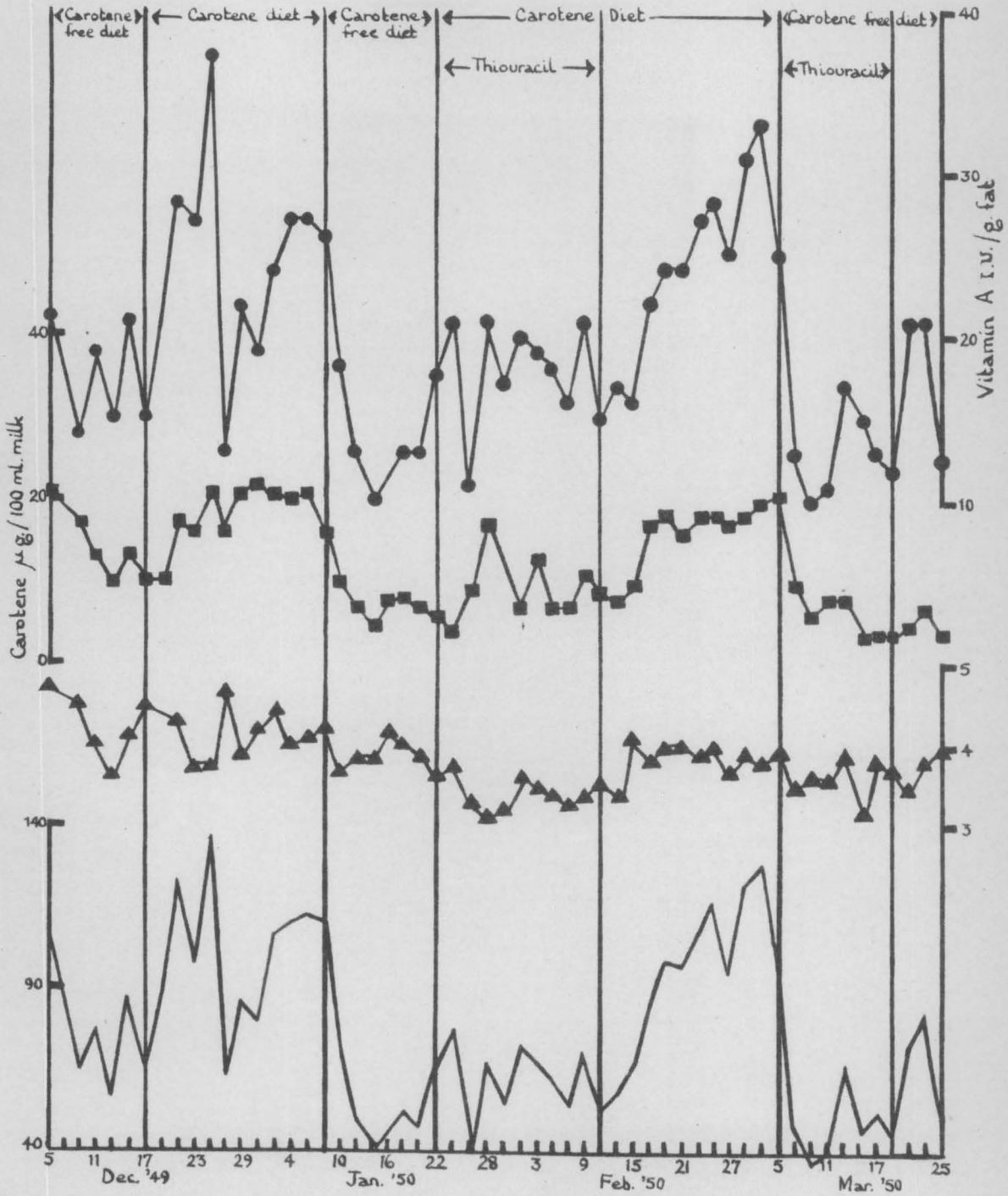


Fig. 22 b

JEAN

Effect of Thiouracil on Carotene and Vitamin A content in Cow's Milk

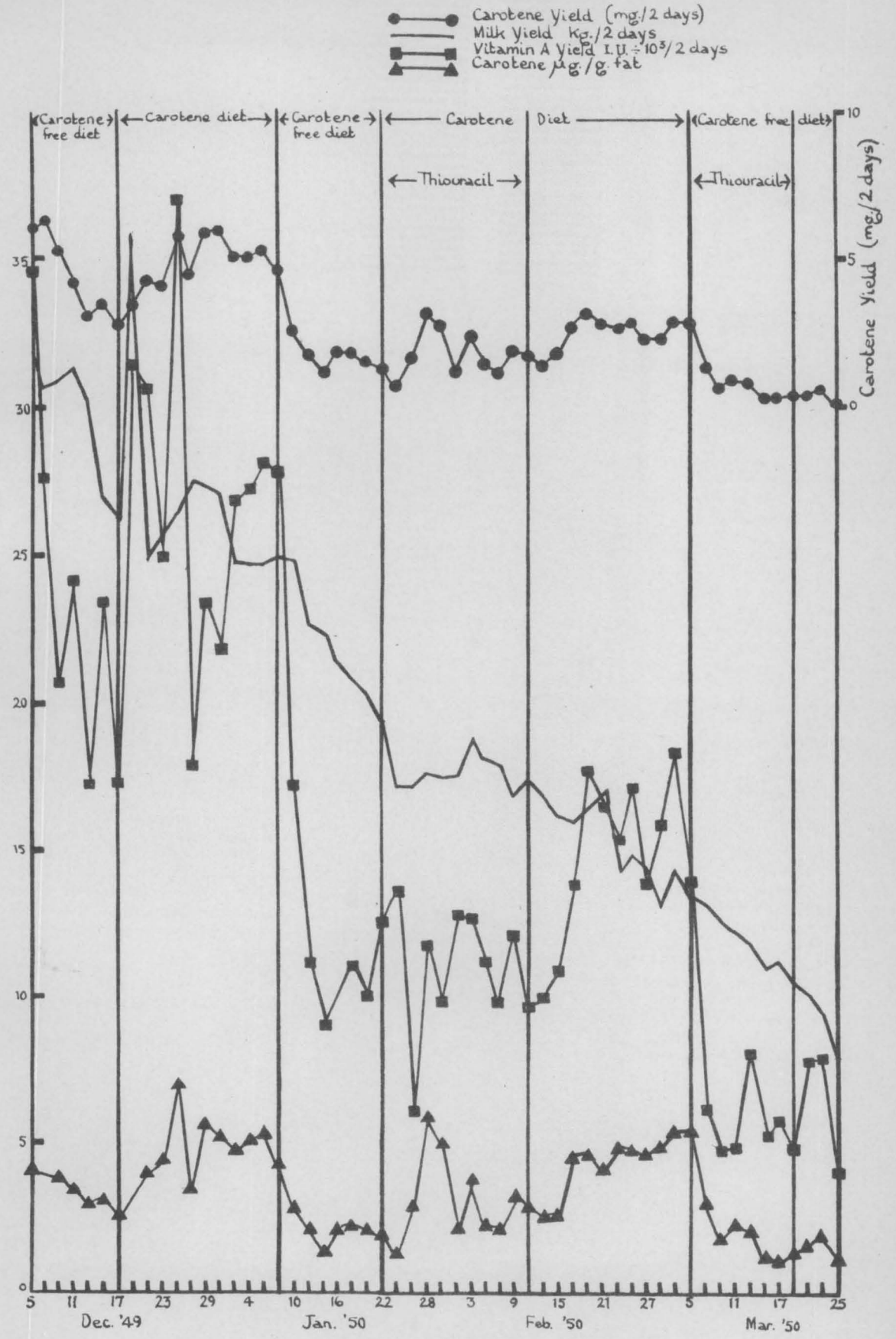
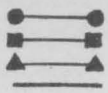


Fig. 23a.

GWYNNETH

Effect of Thiouracil on Carotene and Vitamin A content in Cow's Milk.



Vitamin A I.U./g. fat
Carotene μg./100 ml. milk
% Fat
Vitamin A I.U./100 ml. milk

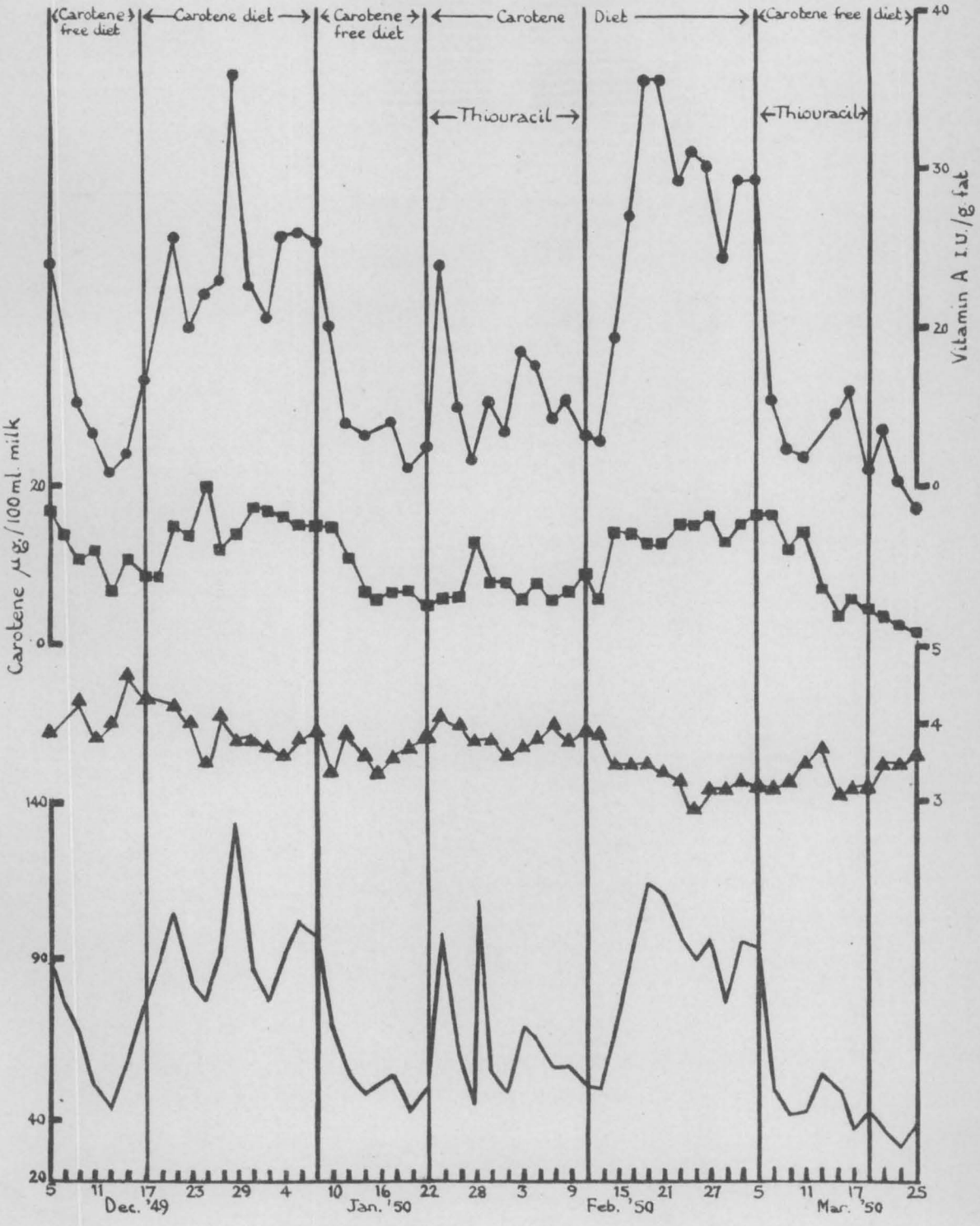
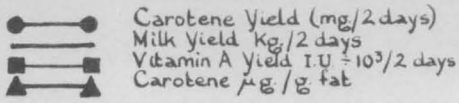


Fig. 23b.

GWYNNETH

Effect of Thiouracil on Carotene and Vitamin A content in Cow's Milk



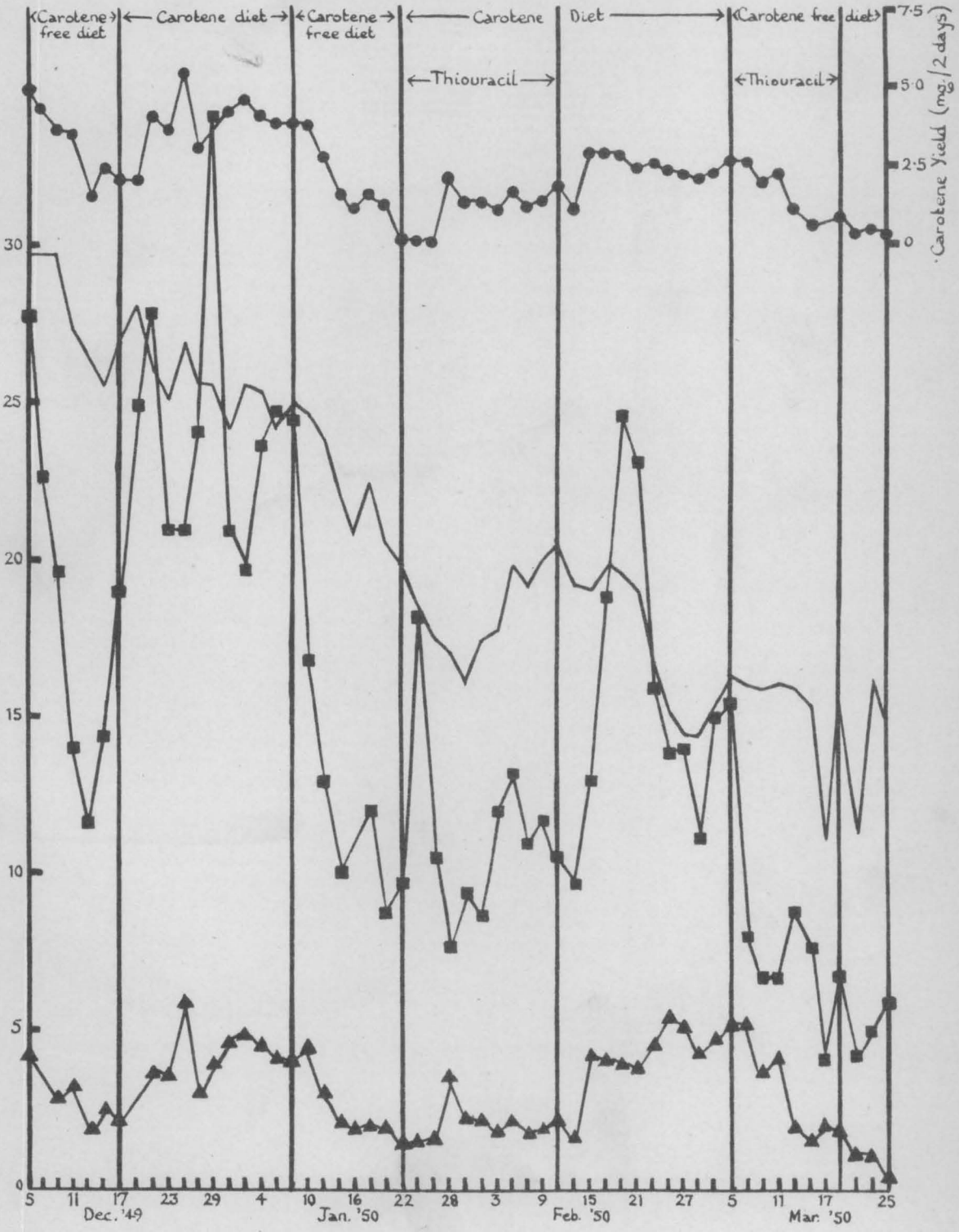


Table 47. The effect of thyroxine and thiouracil on the contents of carotene and vitamin A in cows' milk

Periods	Carotene present (+) or absent (-) in the diet	Control cows		Cows receiving thyroxine in Periods 4 & 6		Cows receiving thiouracil in Periods 4 & 6	
		Dora	Tinker	Delilah	Griselda	Jean	Gwynneth
		Milk yield (kg./2 days)					
1	-	27.5	27.3	28.0	24.0	29.9	28.0
2	+	27.4	26.3	23.1	19.4	26.7	25.7
3	-	24.9	24.1	19.8	15.9	22.4	22.1
4	+	22.6	22.8	22.2	17.0	17.7	18.5
5	+	22.5	22.5	19.1	12.7	15.4	17.3
6	-	21.1	21.5	18.2	11.0	12.0	15.3
7	-	20.3	20.9	20.1	12.2	9.2	14.1
		Fat (%)					
1	-	4.2	4.1	3.9	4.0	4.2	4.2
2	+	3.9	3.6	3.8	4.2	4.1	3.8
3	-	3.6	3.7	3.5	4.1	3.8	3.6
4	+	3.9	3.8	4.2	4.3	3.4	3.9
5	+	3.8	4.0	4.4	4.2	3.9	3.4
6	-	3.7	3.8	4.4	4.0	3.6	3.3
7	-	3.6	4.0	4.3	4.1	3.9	3.5
		Carotene (μg./100 ml. milk)					
1	-	15.1	9.0	12.7	11.8	15.1	12.1
2	+	17.5	14.8	17.4	16.2	18.8	15.4
3	-	9.3	10.1	7.7	8.2	7.9	8.7
4	+	15.0	12.8	23.6	22.5	10.6	8.2
5	+	16.5	15.7	18.8	17.5	17.0	14.1
6	-	11.1	6.6	8.9	9.7	6.7	9.5
7	-	2.9	4.2	3.6	7.2	6.1	3.5
		Vitamin A (i.u./100 ml.)					
1	-	64	59	69	62	79	66
2	+	90	77	88	95	102	95
3	-	60	51	54	55	55	53
4	+	72	70	103	104	62	61
5	+	70	70	80	83	98	92
6	-	52	45	95	110	49	45
7	-	52	48	53	73	70	36

Table 48. The effect of thyroxine and thiouracil on the partition of vitamin A in cows' milk fat

Periods	Carotene present (+) or absent (-) in the diet	Control cows		Cows receiving thyroxine in Periods 4 & 6		Cows receiving thiouracil in Periods 4 & 6	
		Dora	Tinker	Delilah	Griselda	Jean	Gwynneth
		Vitamin A ester (% of total vitamin A)					
1	-	88.6	91.3	90.4	90.5	90.8	91.8
2	+	91.3	93.6	92.7	93.5	91.2	92.1
3	-	89.5	88.9	90.5	90.2	90.9	90.0
4	+	92.5	93.1	92.5	94.0	89.0	88.8
5	+	91.2	93.9	93.8	93.6	91.8	93.2
6	-	88.9	89.1	82.9	79.8	89.6	87.4
7	-	88.6	86.7	87.2	88.3	88.4	89.0
		Vitamin A alcohol (% of total vitamin A)					
1	-	9.3	6.3	7.1	6.4	7.4	7.4
2	+	6.8	4.4	5.5	4.4	6.3	5.5
3	-	9.2	8.0	8.3	7.8	7.7	7.2
4	+	7.2	4.7	5.5	4.4	8.6	8.0
5	+	6.1	4.0	4.9	3.9	6.2	5.1
6	-	8.2	8.6	15.1	18.3	7.3	8.2
7	-	11.3	10.6	11.2	9.1	8.1	8.9

Fig. 24 The Effect of Thyroxine and Thiouracil on the Vitamin A alcohol content in Cow's Milk.

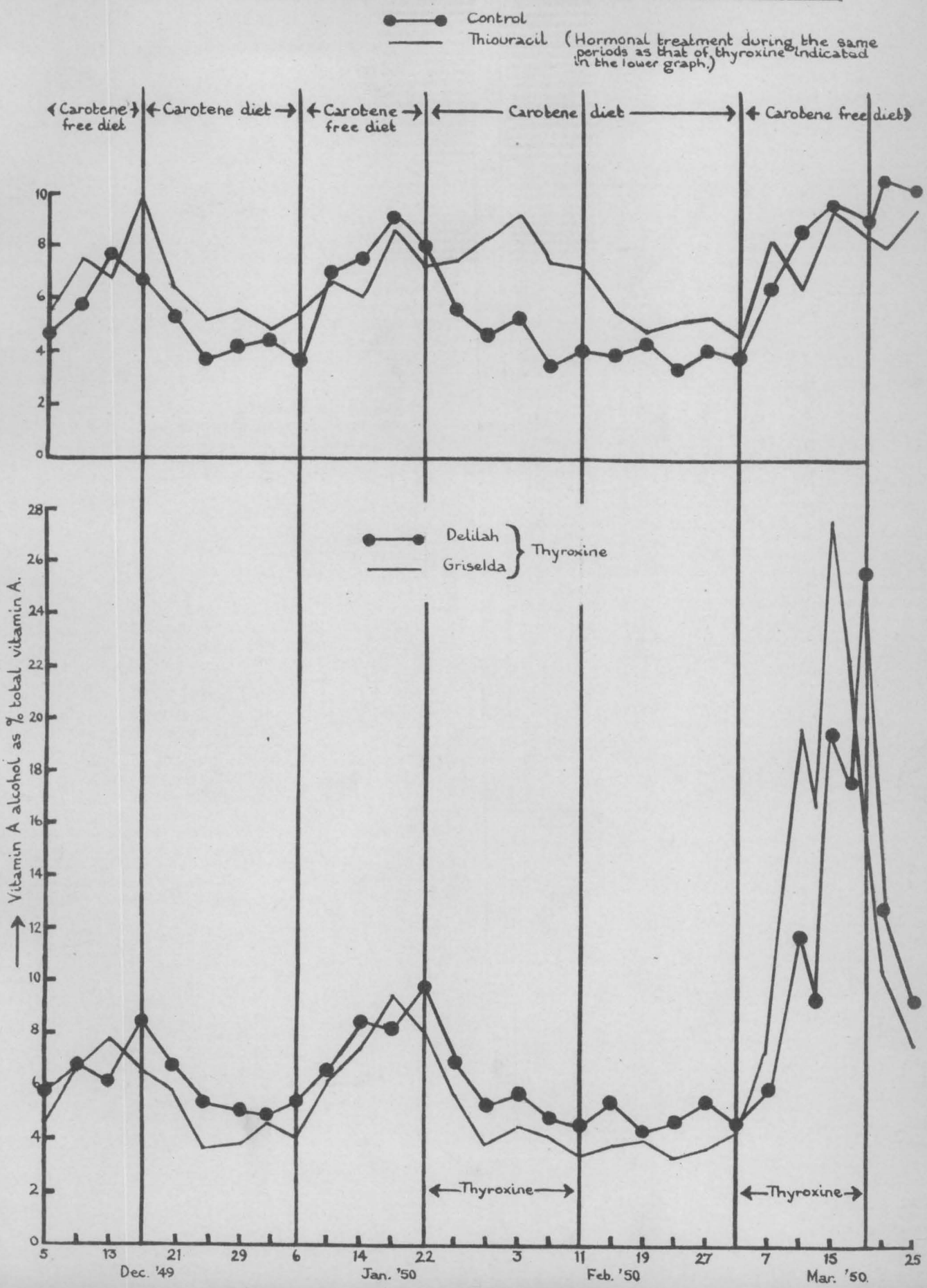


Table 49. The percentage of the total vitamin A potency of the milk fat which is due to carotene

Cows	Periods and treatments	Carotene present (+) or absent(-) in the diet	Vitamin A (i.u./g.fat)	Carotene (μg./g. fat)	Carotene (i.u./g. fat)	Total vitamin A activity (i.u./g.fat)	Proportion of vitamin A activity due to carotene (%)
Dora	1	-	15.2	3.6	6.0	21.2	28.3
	2	+	23.2	4.5	7.5	30.7	24.4
	3	-	16.5	2.6	4.3	20.8	20.7
	4 (control)	+	18.5	3.8	6.4	24.9	25.8
	5	+	18.5	4.3	7.3	25.8	28.2
	6 (control)	-	14.1	3.0	5.0	19.1	26.3
	7	-	14.5	0.8	1.3	15.8	8.5
Tinker	1	-	14.3	2.2	3.7	18.0	20.4
	2	+	21.4	4.1	6.9	28.3	24.3
	3	-	13.7	2.7	4.6	18.3	24.9
	4 (control)	+	18.4	3.4	5.6	24.0	23.4
	5	+	17.4	3.9	6.6	24.0	27.4
	6 (control)	-	11.8	1.7	2.9	14.7	19.7
	7	-	12.1	1.1	1.8	13.9	12.7
Delilah	1	-	17.6	3.3	5.4	23.0	23.6
	2	+	23.1	4.6	7.6	30.7	24.9
	3	-	15.5	2.2	3.7	19.2	19.2
	4 (thyroxine)	+	24.5	5.6	9.4	33.9	27.7
	5	+	18.2	4.3	7.1	25.3	28.2
	6 (thyroxine)	-	21.6	2.0	3.4	25.0	13.5
	7	-	12.4	0.8	1.4	13.8	10.2
Griselda	1	-	15.5	3.0	4.9	20.4	24.1
	2	+	22.6	3.9	6.4	29.0	22.2
	3	-	13.4	2.0	3.3	16.7	19.9
	4 (thyroxine)	+	24.1	5.2	8.7	32.8	26.6
	5	+	19.7	4.2	7.0	26.7	26.1
	6 (thyroxine)	-	27.5	2.4	4.1	31.6	12.9
	7	-	17.8	1.8	2.9	20.7	14.2
Jean	1	-	18.8	3.6	6.0	24.8	24.2
	2	+	24.9	4.6	7.7	32.6	23.5
	3	-	14.4	2.1	3.5	17.9	19.4
	4 (thiouracil)	+	18.4	3.1	5.2	23.6	22.1
	5	+	25.2	4.4	7.3	32.5	22.4
	6 (thiouracil)	-	13.5	1.9	3.1	16.6	18.7
	7	-	18.0	1.6	2.6	20.6	12.7
Gwynneth	1	-	15.6	2.9	4.8	20.4	23.5
	2	+	24.9	4.1	6.8	31.7	21.4
	3	-	14.6	2.4	4.0	18.7	21.6
	4 (thiouracil)	+	15.7	2.1	3.5	19.2	18.2
	5	+	27.1	4.1	6.9	34.0	20.3
	6 (thiouracil)	-	13.7	2.9	4.8	18.5	26.0
	7	-	10.3	1.0	1.7	12.0	13.9

Table 50. The recovery of dietary carotene as carotene and vitamin A in the milk of cows

Cows	Periods and treatments	Carotene intake (i.u. x 10^{-3}/ 2days)*	Vitamin A secreted in milk (i.u. x 10^{-3}/2 days*)			% secretion in milk	Carotene digested (i.u. x 10^{-3}/ * 2days)	% of the apparently digested carotene secreted in milk
			As vitamin A	As carotene	Total			
Dora	2	1630	24.7	7.9	32.6	2.0	960	3.4
	4 (control)	1630	16.4	5.8	22.2	1.4	940	2.4
	5	1580	15.8	6.8	22.6	1.4	870	2.6
Tinker	2	1580	20.2	6.5	26.7	1.7	830	3.2
	4 (control)	1580	16.0	5.9	21.9	1.4	900	2.4
	5	1620	15.7	5.9	21.5	1.3	910	2.4
Delilah	2	1620	20.7	6.7	27.4	1.7	860	3.2
	4 (thyroxine)	1600	21.9	9.1	31.0	1.9	1120	2.8
	5	1600	15.2	6.0	21.2	1.3	920	2.3
Griselda	2	1610	18.4	5.3	23.7	1.5	860	2.8
	4 (thyroxine)	1640	17.8	6.6	24.4	1.5	1200	2.0
	5	1590	10.5	3.7	14.2	0.9	870	1.6
Jean	2	1620	27.0	8.2	35.2	2.2	920	3.8
	4 (thiouracil)	1640	11.1	3.1	14.2	0.9	840	1.7
	5	1550	15.0	4.3	19.3	1.2	830	2.3
Gwynneth	2	1550	24.2	6.6	30.8	2.0	860	3.6
	4 (thiouracil)	1630	11.4	2.5	13.9	0.9	780	1.8
	5	1620	16.0	4.0	20.0	1.2	910	2.2

* For convenience in presenting the data these values are given as thousands of international units.

Effect of thyroxine, thiouracil and stilboestrol on the vitamin A content of goats' milk

The effects of these drugs on the composition of goats' milk are recorded in Figs. 25 to 28 and Tables 51 and 52. The main conclusions were as follows.

(1) It can be seen from Fig.27 that thyroxine caused an initial increase in milk yield which was accompanied by an increase in the percentage of fat in the milk and by an increase of the percentage of vitamin A in the fat. The effect of thyroxine on vitamin A in goats' milk was thus similar to its effect on vitamin A in cows' milk. During the second week of treatment the goats treated with thyroxine showed symptoms of overdosage which caused a reduction in milk yield accompanied by a reduction in the amount of food eaten. In spite of this the vitamin A content (whether calculated per 100 ml. milk or per g. fat) continued to increase (Fig. 27). On cessation of treatment these effects on vitamin A and fat were reversed.

(2) The changes in the composition of the milk of the goat, caused by thiouracil (Fig. 26) were the opposite of those produced by thyroxine but were much less marked.

(3) When stilboestrol was given (Fig. 28) at the same time as thyroxine the well-known drying-off effect of the stilboestrol was predominant so that before the end of the period one of the goats went dry. Before lactation ceased the percentages of fat and of vitamin A in the milk showed very large increases, but the vitamin A

content of the fat was unchanged. On discontinuance of the two drugs the animal again commenced lactating and produced milk of fat and vitamin A contents similar to those of the milk it had been producing when it went dry. A week later, however, the milk reverted to its pretreatment composition. The mean numerical results for all eight goats are shown in Table 51, where it can be seen that the treatment with the drugs affected the milk composition of each member of a similarly treated pair of goats in the same way.

(4) Apart from the fact that there was no carotene in the goats' milk, the treatments with thyroxine and thiouracil affected the recovery of dietary carotene as vitamin A in the milk of the goat in much the same way as it affected the recovery of dietary carotene in cows' milk (Table 52).

(5) As has already been mentioned two of the goats died of hyperthyroidism. The livers and kidneys of these goats and of two other goats not on the experiment were analysed with the results shown in Table 53 together with analyses of the first colostrum. Table 53 shows that in the four livers examined, carotene was present but ranged from 0 in one liver to 0.67 µg. per g. fresh liver. Comparison of these figures for liver carotene with the colostral figures makes it probable that the presence of carotene in colostrum is a reflection of liver reserves but more animals would need to be examined for a valid generalisation. There is a suggestion in

Fig. 25 JUDITH

Vitamin A content in Goats' Milk

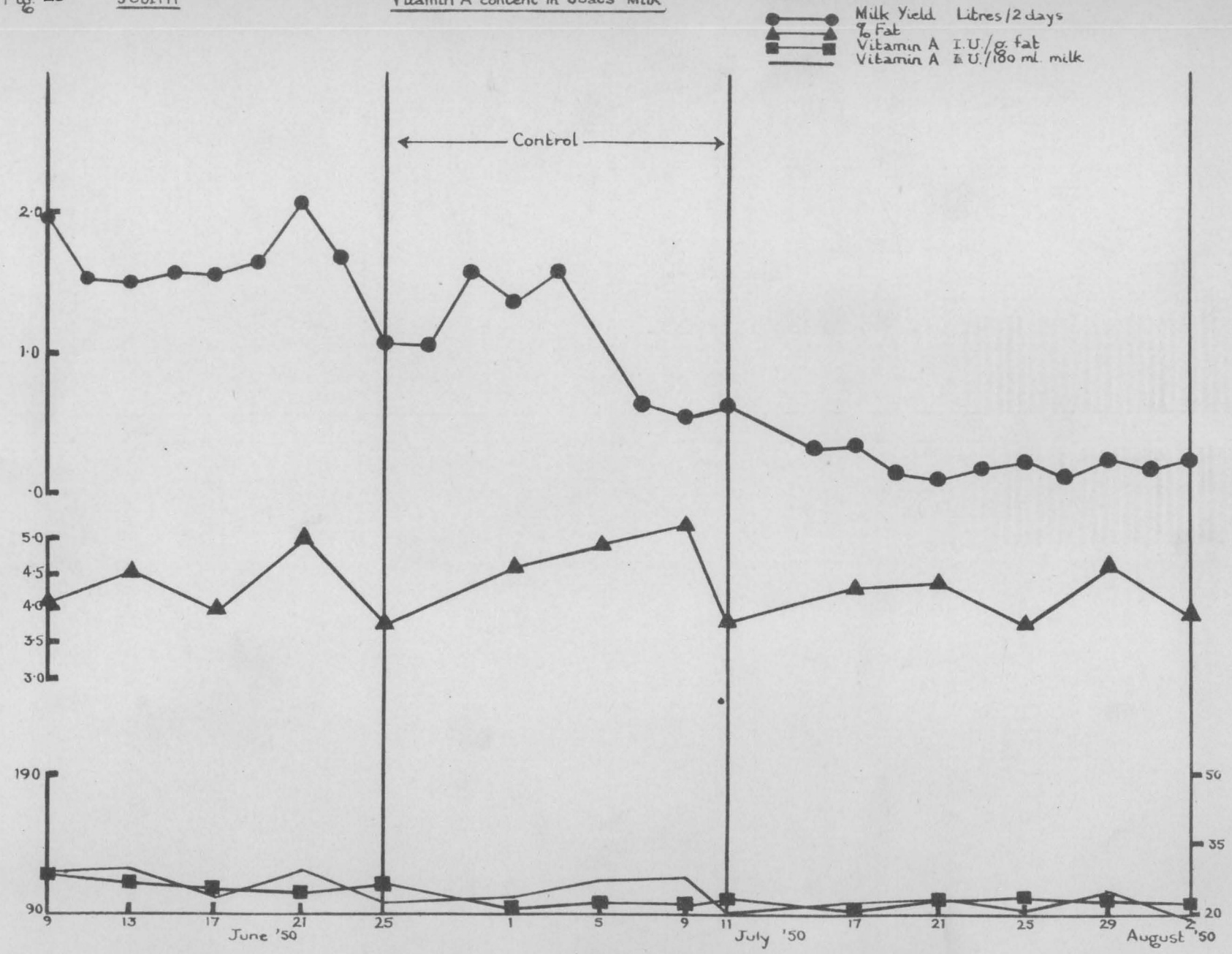


Fig. 26 DIANA

Effect of Thiouracil on the Vitamin A content in Goats' Milk.

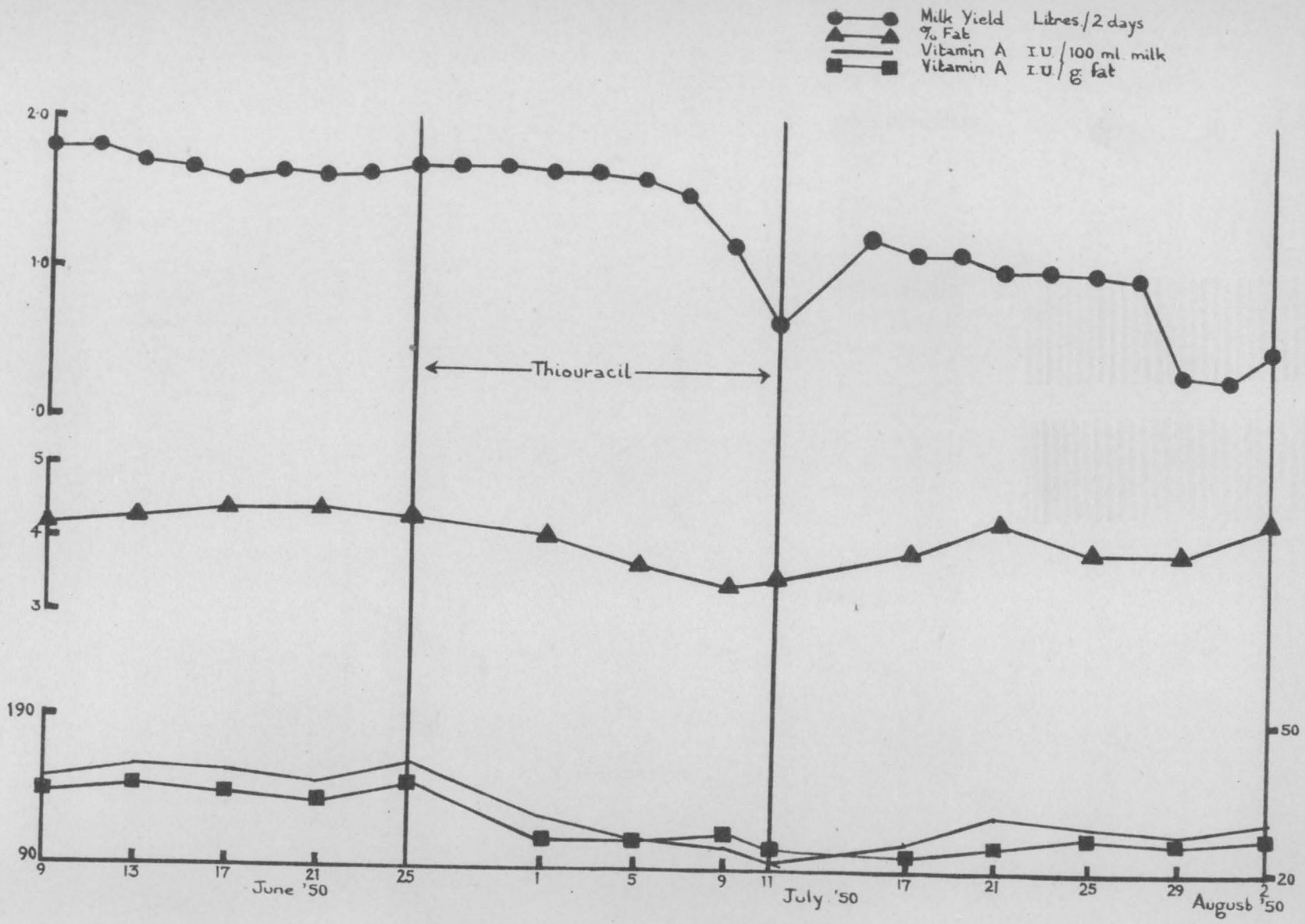


Fig. 27 MAZY

Effect of Thyroxine on the Vitamin A content in Goats' Milk

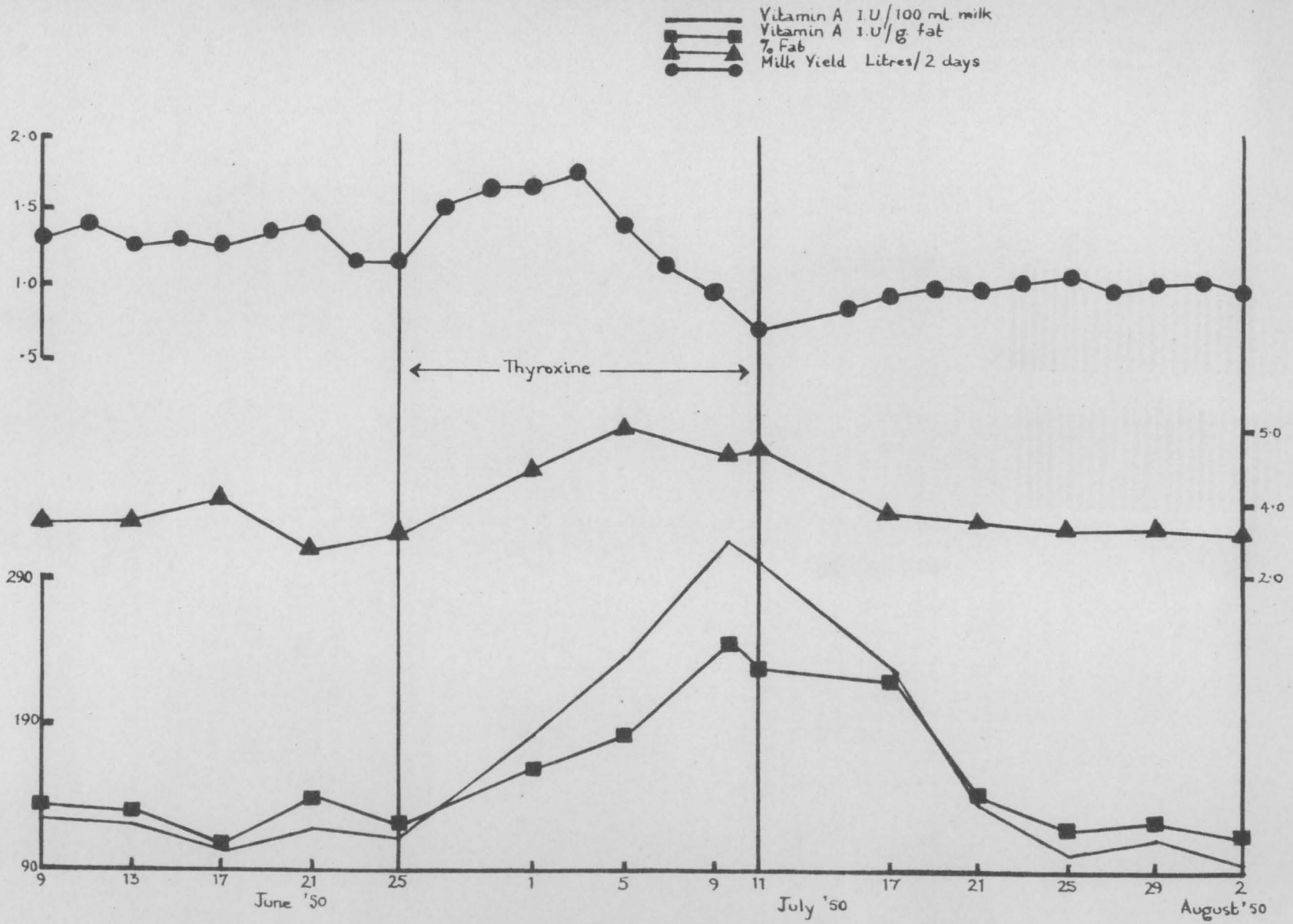


Fig 28 BLUEBELL

Effect of Simultaneous Administration of Thyroxine and Stilboestrol on the Vitamin A content in Goats' Milk.

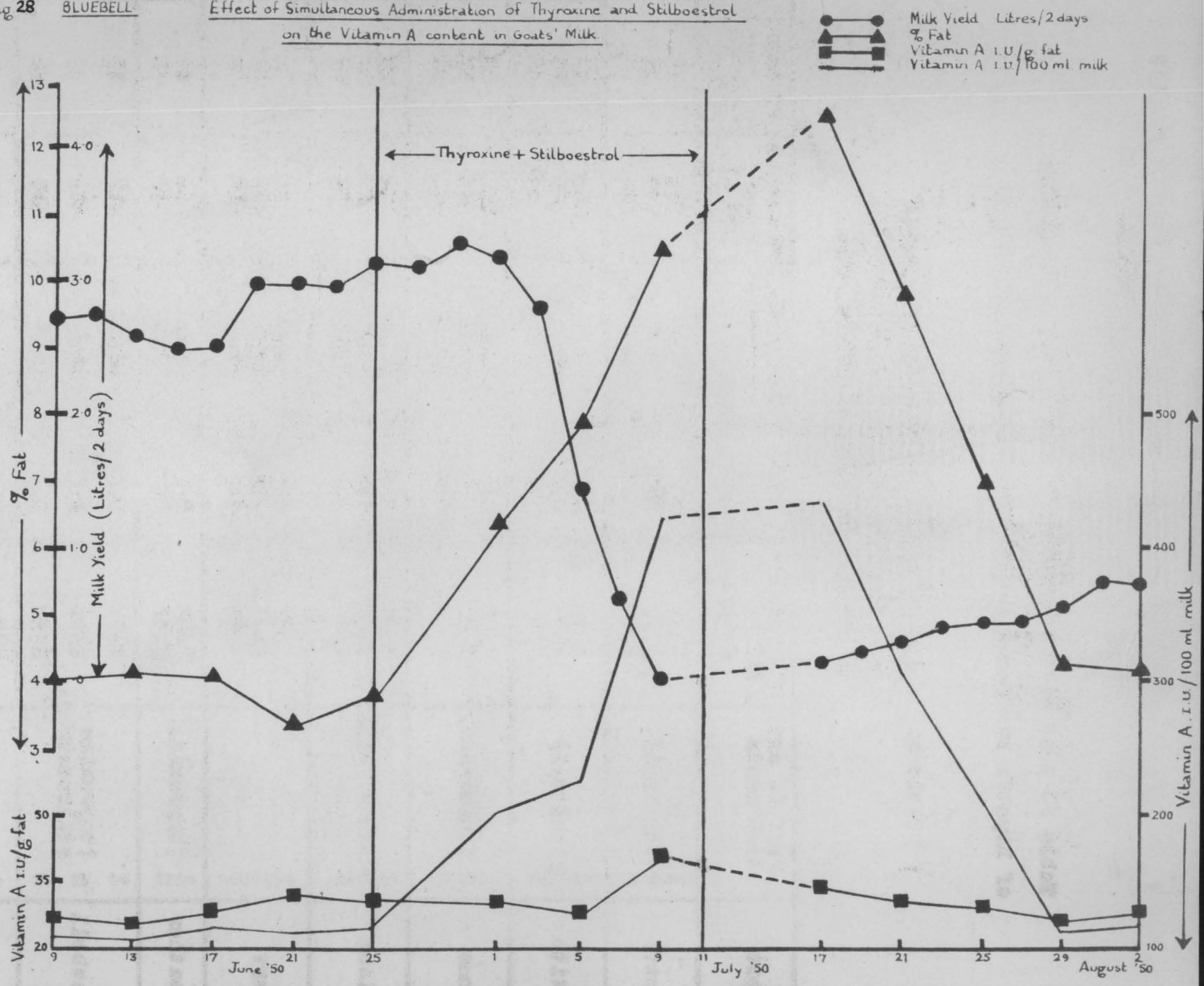


Table 53 that severe hyperthyroidism led to a depletion of hepatic and renal reserves of vitamin A.

Table 51. The effect of thyroxine, thiouracil and stilboestrol on the fat and vitamin A contents of goats' milk

Goat	Periods and treatments	Milk yield (litres/ 2 days)	Fat (%)	Fat yield (g./2days)	Vitamin A (i.u./ 100 ml. milk)	Vitamin A (i.u./g. fat)
Betty	1	2.45	3.1	76.0	179	57
	2 (control)	2.26	3.3	74.7	172	53
	3	1.62	3.3	53.3	168	52
Edith	1	1.54	4.3	66.4	114	27
	2 (control)	1.09	4.6	50.1	107	23
	3	0.23	4.2	9.8	97	23
Anna	1	4.47	4.1	183.1	163	40
	2 (thiouracil)	4.30	3.2	137.5	104	33
	3	3.78	3.6	135.9	108	30
Diana	1	1.67	4.3	71.9	156	36
	2 (thiouracil)	1.44	3.8	54.8	107	30
	3	0.87	4.1	35.6	120	29
Mazy	1	1.35	3.7	50.1	119	32
	2 (thyroxine)	1.33	4.8	63.7	261	55
	3	0.95	3.7	35.1	136	36
Heather	1	2.10	3.1	65.0	134	43
	2 (thyroxine)	0.58	4.4	25.5	225	51
Bluebell	1	3.04	3.9	118.6	112	29
	2 (thyroxine + stilboestrol)	1.92	8.3	159.2	285	34
	3	0.42	7.6	31.7	238	30
Miranda	1	2.24	3.4	76.2	138	41
	2 (thyroxine + stilboestrol)	0.51	9.9	50.4	441	45

Table 52. The recovery of dietary carotene as vitamin A in the milk of goats

Goat	Periods	Carotene intake (i.u. x 10^{-3}/2 days*)	Carotene apparently digested (i.u. x 10^{-3}/2 days*)	Vitamin A secreted in milk (i.u. x 10^{-3}/2 days*)	The proportion of ingested carotene recovered in milk (%)	The proportion of apparently digested carotene recovered in milk (%)
Betty	1	139	88	4.4	3.1	5.0
	2 (control)	124	78	3.9	3.1	5.0
	3	100	65	2.7	2.7	4.2
Judith	1	94	59	1.8	1.9	3.0
	2 (control)	82	50	1.2	1.4	2.3
	3	45	28	2.2	0.5	7.9
Ahna	1	143	98	7.3	5.1	7.4
	2 (thiouracil)	133	71	4.5	3.4	6.3
	3	123	77	4.1	3.3	4.8
Diana	1	138	84	2.6	1.9	3.1
	2 (thiouracil)	109	51	1.5	1.4	3.0
	3	93	60	1.0	1.1	1.7
Mazy	1	122	77	1.6	1.3	2.1
	2 (thyroxine)	70	53	3.5	5.0	6.6
	3	109	63	1.3	1.2	2.1
Heather	1	117	77	2,8	2.4	3.6
	2 (thyroxine)	40	30	1.3	3.3	4.4
Bluebell	1	142	88	3.4	2.4	3.9
	2 (thyroxine + stilboestrol)	87	61	5.5	6.3	8.9
	3	111	64	1.0	0.9	1.6
Miranda	1	114	73	3.1	2.7	4.2
	2 (thyroxine + stilboestrol	12	9	2.2	19.3	23.9

* For convenience in presenting the data these values are given as thousands of international units.

Table 53. Vitamin A in the livers and kidneys of goats

Goat	Cause of death	Liver		Kidney vitamin A (i.u./g.)	β-carotene in the first colostrum (μg./100 ml.)
		Vitamin A (i.u./g.)	Carotene (μg./g.)		
Merle	Unknown	471	0.67	81	35
Jean	"	452	0.09	77	19
Heather*	Hyper-thyroid-ism	369	0.34	38	28
Miranda*	" "	253	-	9	12

* These goats ate very little carotene containing ration from the time they became hyperthyroid until their death.

Discussion

Carotene metabolism of cows and goats

The experimental results presented in this chapter show that the thyroid plays an important role in the absorption of carotene from the alimentary tract in lactating cows and goats and in the subsequent secretion of vitamin A and carotene in their milk. In Section 1 of this chapter it was shown that the goats absorbed more carotene than did the cows when fed similar amounts of carotene (1.1 - 1.2 mg./kg. body weight). The higher digestibilities in the goat may be related to the naturally higher thyroid activity of the goat, for Schultze & Turner (1945) showed that the goat's thyroid gland is more active than the cow's. The results obtained by injecting thyroxine or the anti-thyroid drug, thiouracil, into lactating cows and goats provide further independent evidence in favour of the view that the goat has the more active thyroid, for they demonstrate (a) that thyroxine increased the power of the cows to digest carotene more than it increased the power of the goats to digest carotene and (b) that, conversely, thiouracil decreased the power of the goats to digest carotene more than it decreased the power of the cows to digest carotene.

Cama & Goodwin (1949a) found that thiouracil increased while desiccated thyroid reduced the faecal carotene in rats. In their experiments Cama & Goodwin gave the drugs orally. From the experiments of Glover,

Goodwin & Morton (1948) and of Thompson, Ganguly & Kon (1949) it would be reasonable to suppose that orally administered drugs, since they reach the intestinal mucosa from the lumen, might be more active in affecting an enzyme system in the intestinal wall than the same drugs injected. The present results show, however, (1) that the effects of both thyroxine and thiouracil are independent of the route of administration of the drugs, (2) that the effects occur in the two genera - Bos and capra, and (3) that the large fibre intake of the ruminant does not inhibit these effects. If the sole site of conversion of carotene to vitamin A is the intestinal wall (Glover et al., 1948; Thompson et al., 1949), these results demonstrate that the biochemistry of the intestinal mucosa of the ruminant can be influenced by thyroxine.

The hypothesis, that the greater digestibilities found in thyroxine treated animals are due to greater absorption of carotene and greater conversion of carotene to vitamin A is supported by the finding that vitamin A ester in milk was increased by thyroxine (Tables 47 & 51). This indirect evidence, indicating as it does that the rate of conversion of carotene to vitamin A is accelerated by thyroxine and slowed down by thiouracil, weakens the validity of applying the equation.

0.6 μg. β-carotene = 1 i.u. vitamin A

indiscriminately to all species or even to individuals within one species. From the results reported in the

present chapter it may be seen that in extreme hyper- or hypo-thyroidism the amount of carotene converted or made available for conversion may vary widely.

A possible criticism of the present experiments is that the failure of carotene to appear in the faeces does not necessarily mean that more had been absorbed since part of the carotene might simply have been decomposed in the alimentary tract. The observations of Seshan & Sen (1942b) and of Goodwin & Gregory (1948), however, argue against this criticism. Goodwin & Gregory (1948) could demonstrate no loss of carotene on incubation at 37° with the intestinal contents of the rabbit, in spite of the fact that they showed that this animal can digest 86.4% of its dietary carotene. Seshan & Sen (1942) similarly failed to find evidence of destruction of carotene in the gut, no loss occurring when carotene was incubated with cattle faeces. These observations in conjunction with the present findings that the milk vitamin A is increased by thyroxine make it likely that the greater digestibility after thyroxine is due to greater absorption and not to greater destruction in the alimentary tract.

The decreased chromium concentration (Table 40) in the faeces of cows during thyroxine treatment, indicates that the digestibility of dry matter decreased during this period, for no variations occurred in the intakes of either concentrates or roughages. A similar observation was made earlier by Owen (1948a). The digestibility of carotene was thus increased at the same time as that of the bulk of the dry matter was decreased. This observation is only to be expected if

the chief site of conversion of carotene to vitamin A is the intestine.

Carotene and vitamin A in milk of cows and goats

It is important to note that the milk fat and body fat of the cow contain carotenoids and are consequently yellow while those of the goat are colourless. The administration of thyroxine or thiouracil to goats failed to make them secrete carotene in their milk fat. In established lactation no measurable quantity of carotene appeared in the goats' milk. An interesting observation made with some of the goats was that the first day's colostrum was coloured yellow with carotenoids including β-carotene (Table 53). During the days which followed the presence of traces of β-carotene could still be demonstrated chromatographically. These phenomena were noted even in the colostrum of a goat which had been giving some carotene-free milk just prior to parturition. Carotene was found to be present in variable amounts in goat liver (Table 53). The goat Marie which had the greatest concentration of carotene in its liver, also secreted the most carotene in her colostrum (35 μg./100 ml.). It therefore seems that there is some hormonal effect operating at parturition which causes carotene to appear even in goats' milk, but it is justifiable to conclude from the results recorded in this thesis that whatever the hormone may be it is not thyroxine. Cows receiving thyroxine secreted more carotene in their milk, while control cows secreted more than the thiouracil ones. The larger

concentration of carotene in milk from cows treated with thyroxine provides no explanation of the claim of Fellenberg & Grüter (1932), that thyroidectomised goats gave yellow milk.

The results of hormone treatment on the recovery of ingested carotene in the milk as carotene and vitamin A show that in cows the recovery was increased by thyroxine and decreased by thiouracil (Table 50). When however, the recoveries were calculated as percentages of the apparently digested carotene, the recoveries in the milk of thyroxine cows were less during treatment than they had been during the previous carotene period without treatment. This may indicate that the larger quantity of vitamin A presumably formed under the influence of thyroxine was only partly secreted in the milk while a portion was used to replenish the hepatic stores, since the cows were receiving a carotene-free diet immediately before this period. In goats, a larger apparent recovery of dietary carotene in the milk as vitamin A was found in the animals receiving thyroxine or thyroxine plus stilboestrol (Table 52). These effects were observed when the recoveries were calculated as percentages of either the ingested or the digested amounts. The carotene intakes of the hyperthyroid goats were very low and the highest apparent recovery of 24% observed in a goat receiving thyroxine plus stilboestrol was probably enhanced by hepatic depletion.

The fact that vitamin A in the milk of cows was lower when thiouracil was given with a carotene-free

diet than it had been on the same diet without thiouracil can be explained by the observation of Kelly & Day (1948) who stated that thiouracil retards the rate of depletion of hepatic stores.

Fig. 24 shows, that whenever a carotene-free diet was given to cows, there was a small but significant increase in the alcoholic form of vitamin A in their milk. When the process of depletion was further accelerated by thyroxine still larger amounts of vitamin A alcohol appeared in the milk of cows on the carotene-free ration. Nearly all of the vitamin A in the milk of ruminants is in the ester form while that in the blood is in the alcoholic form. It has therefore been supposed that vitamin A is esterified in the mammary epithelium. On such a hypothesis it is difficult to explain the appearance of more of the alcoholic form of vitamin A in the milk of cows deprived of carotene, or the still larger increase exhibited by such cows when given thyroxine.

Summary

The effects of thyroxine and thiouracil injections on the metabolism of carotene and on the secretion of vitamin A in the milk have been investigated in lactating cows and goats. The following were the main results:

1. Subjection of lactating cows to a diet free from carotene reduced faecal carotene to a minimal value of 100 µg. per 100 g. dry faeces in 6-8 days, from an

initial figure of about 3 mg./100 g. when the animals were ingesting carotene. Re-instatement of the carotene regime caused a rapid reappearance of faecal carotene.

2. The rate of reappearance of faecal carotene in cows after re-instatement of the carotene-diet was markedly accelerated by thiouracil and markedly decelerated by thyroxine. The mean rate of reappearance was 277 μg. per day in six cows during a period of three weeks. When the drugs were administered and the same diet fed the rates of reappearance were appreciably higher in thiouracil cows and appreciably lower in the thyroxine cows.

3. The digestibility of carotene by cows was markedly increased by thyroxine and markedly decreased by thiouracil. The digestibility of carotene in goats was also similarly affected by the drugs but the increase due to thyroxine was bigger in the cows than in the goats. By contrast the decrease of digestibility due to thiouracil was bigger in the goats than in the cows.

(4) It was found that goats normally secrete milk fat which is richer in vitamin A than that of Ayrshire cows fed the same amount of carotene per unit body weight. No free vitamin A alcohol was found in goats' milk. In cows' 6% of the vitamin A was free.

5. The carotene and vitamin A contents of milk were

increased when thyroxine was given to cows ingesting carotene. On the same diet thiouracil treatment caused a decrease in the contents of both carotene and vitamin A.

6. When thyroxine treatment was given to cows on a carotene-free diet, there was the usual decrease in the carotene content of the milk but marked maxima were recorded for the vitamin A content of the milk. It was found that these maxima corresponded to large increases in the alcoholic form of vitamin A. When thiouracil was given to cows on a carotene-free diet both carotene and vitamin A in the milk dropped rapidly. On cessation of treatment but continuing the same diet there was a transient increase in vitamin A without any change in carotene.

7. A small increase in the recovery of dietary carotene as milk vitamin A occurred in thyroxine-treated cows. Thiouracil caused a decrease in this recovery.

8. Hyperthyroidism in the goats caused a marked increase in the fat content of the milk. The vitamin A content of fat was also markedly increased. On cessation of treatment the vitamin A content of the fat returned to normal. Thiouracil caused a decrease in the vitamin A content of goat butter fat.

9. Carotene was found to be present in variable amounts in goats' colostrum but no carotene appeared in the milk of hyper- or hypo-thyroid goats.

CHAPTER IV

The Chemical Composition of Human Milk, with particular reference to the contents of fat, solids-not-fat, protein, carotenoids and vitamin A, and the relation of phosphatase to the partition of phosphorus and aneurin

The best recognised substitute for human milk for infant feeding is the milk of other mammalian species, such as cow, goat, buffalo and sheep. Formulae have from time to time been proposed for diluting and adjusting cow's milk to give a mixture similar in composition to human milk. A study made by the American Medical Association (Grulee, Sanford & Herron, 1934) reported the incidence of disease and the death rate of 20,061 infants who were breast fed, partially breast fed or artificially fed. Only 8.5% of the infants studied were artificially fed but their death rate was ten times as high as that of the breast fed infants.

In recent times it has become increasingly apparent that the qualitative nature of the nutrient is as important as its quantity. There is evidence that milk tends to be ideally suited to the conditions of the young of the animal producing it (Brody, 1945; Kay, 1937) so that faster growing animals tend to be provided by their mothers with milk richer in protein, minerals and B-vitamins, while animals born in a very cold environment tend to receive milk very rich in fat and sometimes completely lacking in carbohydrate (Davies, 1936; Kay, 1937).

Knowledge of the qualitative and quantitative differences between the chemical composition of human and cow's colostrum and milk is therefore invaluable. By such knowledge it should be possible to judge the relative merits of various popular methods of 'humanising' cow's milk. Work so far published on this subject (Kon & Mawson, 1950; Clements, 1949) shows that human milk is more variable in composition than cow's milk. That the domestic cow is the result of many hundreds of years of selection for milk production may partly account for this difference, but, as in other fields of human variability, it would, in the absence of special researches directed towards that end, be mere speculation to attempt to assess the relative effects of heredity and environment on human milk secretion.

Comparisons of the compositions of the milk of various species must, however, be treated with reserve partly because the young of different species undergo widely varying degrees of pre-natal development, but chiefly because except in the cow, the goat and the sheep, all of which provide milk for cheese manufacture, the published figures for milk composition are not based on a sufficient number of comparable samples. It is also very important to study the nature of some of the minor constituents of the milk which have important nutritional properties.

A detailed study of the composition of the milk and colostrum of cows and goats, as affected by hormonal and nutritional influences has been reported

in previous chapters. The present work was undertaken to see whether changes occurring in human milk at the inception of lactation were at all comparable with those occurring in cow's milk immediately after parturition or after hormone treatment. At the same time the partitions of carotenoids and of vitamin A were also studied. Some typical results showing the partition of phosphorus and aneurin in goat's milk have been included in the present Chapter for comparison.

Experimental

Milk samples from mothers delivered in the Aberdeen Maternity Hospital were obtained through the courtesy of Dr Bertine Crammond who was responsible for the collection of the samples and their despatch to the Hannah Institute for analysis. Milk was not collected during summer months so that possible changes during transit were minimized. The samples sent in the evening usually arrived the following morning and were in good condition when received in the laboratory. Analyses were started immediately and completed within the next 48 hours.

Methods of Analysis

Fat, solids-not-fat and protein (N x 6.38) were determined by the routine procedures used for cow's milk. The methods for the partition of phosphorus and aneurin have already been described in Chapter I (p.14). In this experiment inorganic phosphorus was estimated

by applying the method of Fiske & Subbarow (1925) directly to the unhydrolysed tricholoroacetic acid extract. Free aneurin was determined by Jansen's method in skimmed milk. Total aneurin was determined after incubation of skimmed milk with takadiastase (Parke, Davis & Co.) according to the method of Houston, Kon & Thompson (1940). Phosphatase was estimated by Neave's modification of the method of Kay & Graham (1935) as outlined by Kay, Aschaffenburg & Neave (1939). The lengthier test (24 hr. incubation at 37°) was used for human and goat's milk because of very low phosphatase activity in their milk.

In estimating carotenoids and vitamin A, it was found that in addition to β-carotene and xanthophylls, human milk fat contained appreciable amounts of α-carotene and, as Thompson, Kon and Mawson (1942) had shown before, it also contained lycopene. These substances were therefore separated chromatographically. The procedure for separating carotenoids, vitamin A ester and vitamin A alcohol was essentially as described by Ganguly, Kon & Thompson (1947). The method was suitably modified as follows to include lycopene and further to partition the α and β forms of carotene.

All solvents used were purified. Diethyl/ether was freed from peroxide by treatment with stannous chloride followed by distillation. Light petroleum and n-hexane were freed from aromatic hydrocarbons by treatment with H_2SO_4 (density 1.84 g./ml.), followed by distillation. Acetone was redistilled. Ethanol was

freed from aldehydes by treatment with KOH and $AgNO_3$ followed by distillation just before use.

Fat was extracted from the milk by the method of Olson, Hegsted & Peterson (1939). To 50 ml. of milk taken in a 250 ml. separating funnel, 7.5 ml. of 35% ammonia were added. After vigorous shaking the contents were allowed to stand for 3-5 min., after which 30 ml. of ethanol, 35 ml. freshly distilled diethyl ether and 15 ml. light petroleum (B.P. 40-60°) were added successively, the mixture being shaken after each addition. The separating funnel was then allowed to stand till the layers were cleanly separated. The lower layer was run off into a flask and the top layer collected in another flask. The lower layer was transferred back into the separating funnel and again extracted with 25 ml. diethyl ether and 10 ml. light petroleum. The two extracts were combined and allowed to stand in the separating funnel for 30 min. Any aqueous layer which separated was drawn off. After having been washed twice with 50 ml. portions of warm tap water with gentle shaking so as to avoid any formation of emulsion, the ether layer was dried over sodium sulphate and poured into a wide-mouthed flask. The sodium sulphate was washed twice with small volumes of ether which were added to the flask. The ether was removed by evaporation under reduced pressure on a water bath. The fat so obtained was taken up in 5 ml. n-hexane and chromatographed in a 4 x 1 cm. column of Savory & Moore's alumina. α- and β-carotene, lycopene and vitamin A ester for the most

part passed through with the fat. Thereafter 20 ml. 3% acetone in n-hexane served to complete the elution but kept the vitamin A alcohol still in the column. The eluates of the same sample were combined (Fraction 1). Vitamin A alcohol was then eluted with 20 ml. 8% ethanol in n-hexane. This eluate was evaporated to dryness at 70° under reduced pressure. As soon as the solvent had all evaporated, the residue was taken up in 5 ml. n-hexane and its absorption of ultraviolet light at 328 mμ determined in a Unicam Spectrophotometer.

Fraction 1 was evaporated under reduced pressure and the fat saponified with 1 g. KOH and 20 ml. alcohol by boiling for 5 min. The contents were diluted with three volumes of ice-cold water and the mixture allowed to cool in the dark. The solution was then transferred to a separating funnel and extracted first with 50 then with 30 and finally with 20 ml. of peroxide-free diethyl ether. The extracts were combined and washed with three 50 ml. lots of distilled water, shaking being gentle with the first and vigorous with the second and third lots. It was found necessary sometimes to use a little N HCl in the second washing to remove the alkali completely. The final washing was tested to make sure that the extract was free from acid or alkali. The extract was dried over sodium sulphate evaporated in a wide-mouthed flask under reduced pressure and then taken up in 5 ml. n-hexane prior to being chromatographed again on a 4 x 1 cm. column of alumina. α- and β-carotene were eluted with 3% acetone in n-

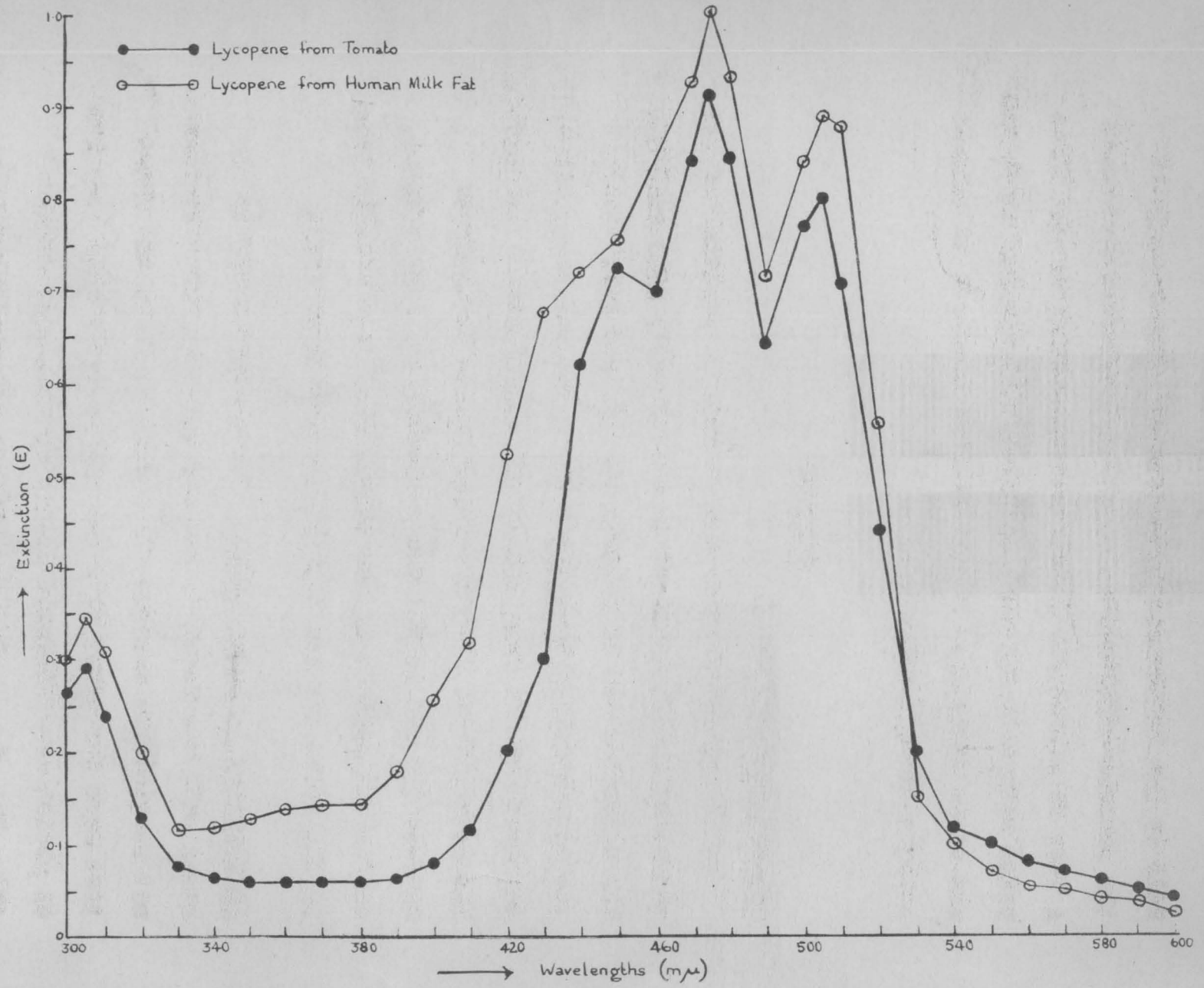
hexane. The eluate was made up to a suitable volume after evaporation under reduced pressure and read in the spectrophotometer at 451 mμ using a tungsten lamp. Vitamin A ester of the original fat (now converted to vitamin A alcohol by the saponification) remained absorbed in the column along with the lycopene. The lycopene and vitamin A alcohol were eluted together by 10% acetone in n-hexane. Lycopene was estimated in the spectrophotometer by its absorption at 505 mμ. $E^{1\%}_{1cm.}$ in n-hexane is 2000, (Morton, 1942).

In this solution it was not possible to estimate vitamin A because the ultraviolet absorption of lycopene itself overlapped that of vitamin A. As inspection of Fig. 29 shows, the absorption curve of lycopene has a distinct bend near 328 mμ, so that the conditions defined by Morton & Stubbs (1946) for correction for irrelevant absorption did not obtain. By the following method, however, lycopene and vitamin A were separated. When fat containing vitamin A and carotenoids is chromatographed in n-hexane, lycopene can be held on the column of alumina while the elution of carotene and vitamin A ester is completed with 3% acetone in n-hexane, provided a longer column is used and the concentration of fat on the column of alumina is not too large. By adjusting the length of the column and decreasing the amount of material taken (so as to reduce the concentration of fat on the column) vitamin A ester, free from lycopene, can be eluted. Accordingly, for the estimation of vitamin A ester a

separate sample was extracted from the milk as already described and chromatographed in two portions, each in a 6 x 1 cm. column of alumina. By thus reducing the amount of fat per column the slower moving band of lycopene was retained while α- and β-carotene and vitamin A ester were eluted with the fat. Further elution of these columns with 3% acetone in n-hexane did not result in any appreciable movement of the lycopene band. The pair of eluates of the same sample consisting of carotene and vitamin A ester were combined and evaporated under reduced pressure. The fat was saponified. Carotene and vitamin A were extracted with ether as before and the final ether extract evaporated to dryness. The residue was taken up in 5 ml. n-hexane and chromatographed again. α- and β-carotene were eluted with 3% acetone in n-hexane, and vitamin A ester, now converted to vitamin A alcohol, was eluted separately with 8% ethanol in n-hexane.

After evaporation under reduced pressure the extracts were dissolved in a suitable volume of n-hexane and read on the spectrophotometer, the vitamin A at 328 mμ and the carotene at 451 mμ. Partition of α- and β-carotene was accomplished by adsorption on a column of magnesia followed by elution by a 1% solution of acetone in n-hexane. It was assumed that $E^{1\%}_{1cm.}$ 451 mμ in n-hexane for the mixed carotenoids was 2400. Actually, however, this constant is 2500 for β-carotene and 2200 for α-carotene. $E^{1\%}_{1cm.}$ for a sample of β-carotene supplied by British Drug Houses, Ltd., was found to be

Fig. 29 Absorption Spectrum of Lycopene from Tomato and Red Pigment from Human Milk dissolved in n-hexane.



2415 in the Unicam Spectrophotometer used throughout the present experiments. All the readings for vitamin A were corrected for irrelevant absorption by the three point method of Morton & Stubbs (1946) already mentioned and their conversion factor of 1760 was used in calculating vitamin A potencies.

Results

General Composition

Table 54 shows the average composition of all the milk samples obtained on the same day post partum. The concentration of protein (0.63%) was very small on the second day post partum but became much bigger (2.01%) the next day and remained relatively constant thereafter. The averages for fat percentage varied irregularly between 2.5 and 3.9%. The percentage of non-fatty solids was maximal on the second day, considerably less on the third day and fairly constant thereafter.

Phosphatase in human milk. Phosphatase activity in human milk was found to be very low. It was therefore not possible to measure the enzyme titre by the short test used for cows' milk because no measurable quantity of phenol was liberated during 10 min. incubation at 47°. The figures for phosphatase recorded in Tables 55, 57, 58, for human milk and in Table 64 for goats' milk, are therefore not comparable numerically with that of cow's milk recorded in Chapter I.

Phosphatase content of the human milk was initially large but decreased during the first days of

lactation. On the twentyeighth day post partum, however, it was increased to a figure slightly higher than that recorded on the second day post partum (Table 58)

Phosphorus partition. The phosphorus contents and the phosphorus partitions of 34 samples of colostrum and transitional milk from the 2nd to the 12th day post partum are shown in Table 55. The coefficients of correlation of ester P with phosphatase are shown in Table 63 together with other coefficients of correlation to which reference will be made later.

Table 55, in which average figures are presented, and Table 63 both show that ester-phosphorus tended to be greater when phosphatase was smaller and vice-versa. This correlation had been noted before in the lactating cow (Chapter I) and was one of the reasons for doing the present work. The relationship between phosphatase and ester-phosphorus is shown in Fig.30. The negative correlation between ester phosphorus and phosphatase was closer than would have been expected from an inspection of the standard errors shown in Table 55. This was because averaging of samples belonging to the same day hid the fact that the correlation held between mothers on a given day as well as from day to day. The analysis of variance (Table 56) showed a significant curvilinearity of the relationship. A curve was therefore fitted by the method of least squares.

Lipid phosphorus (Tables 57 and 63) was also negatively correlated with phosphatase, while inorganic

Fig 30.

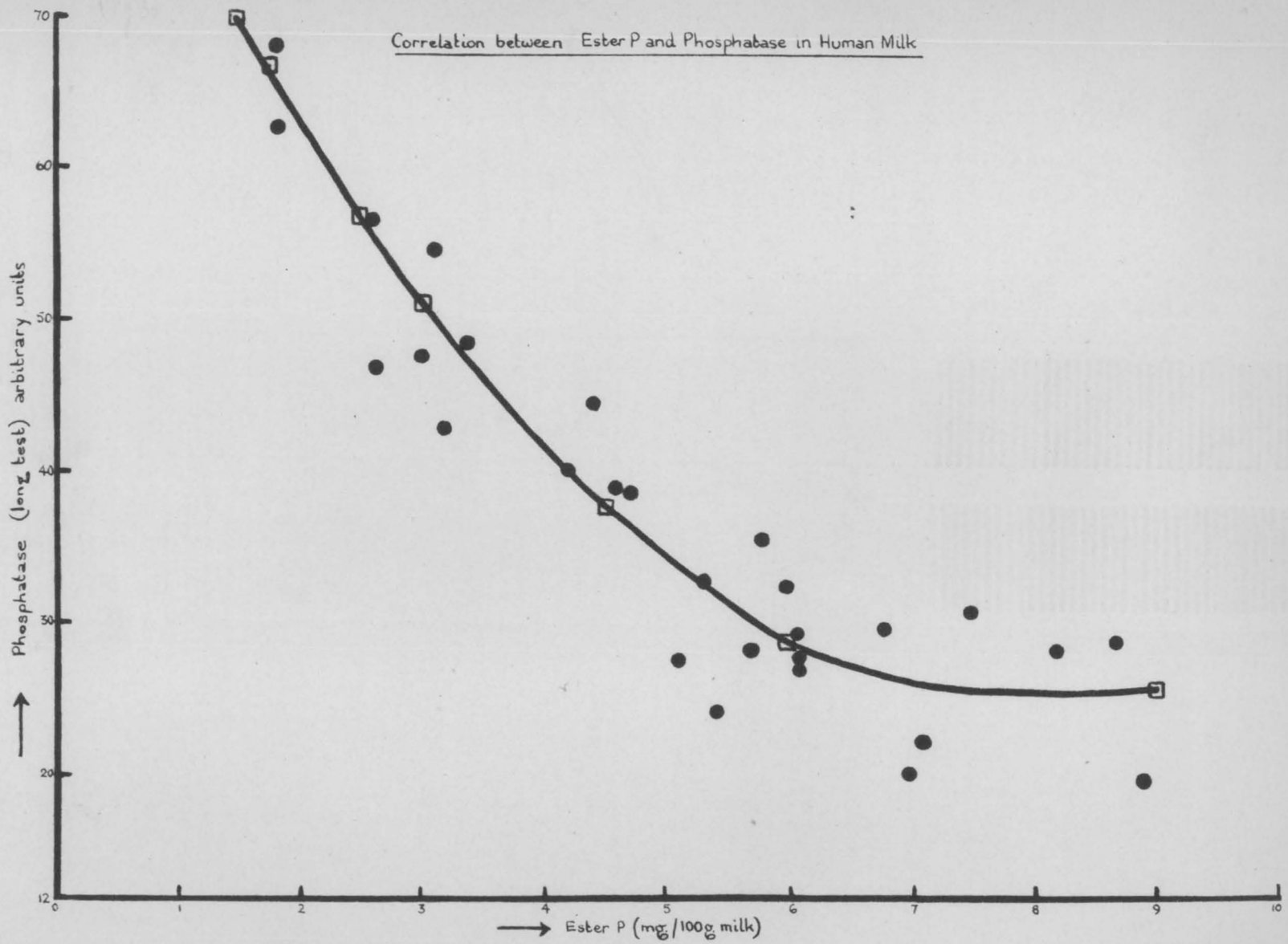
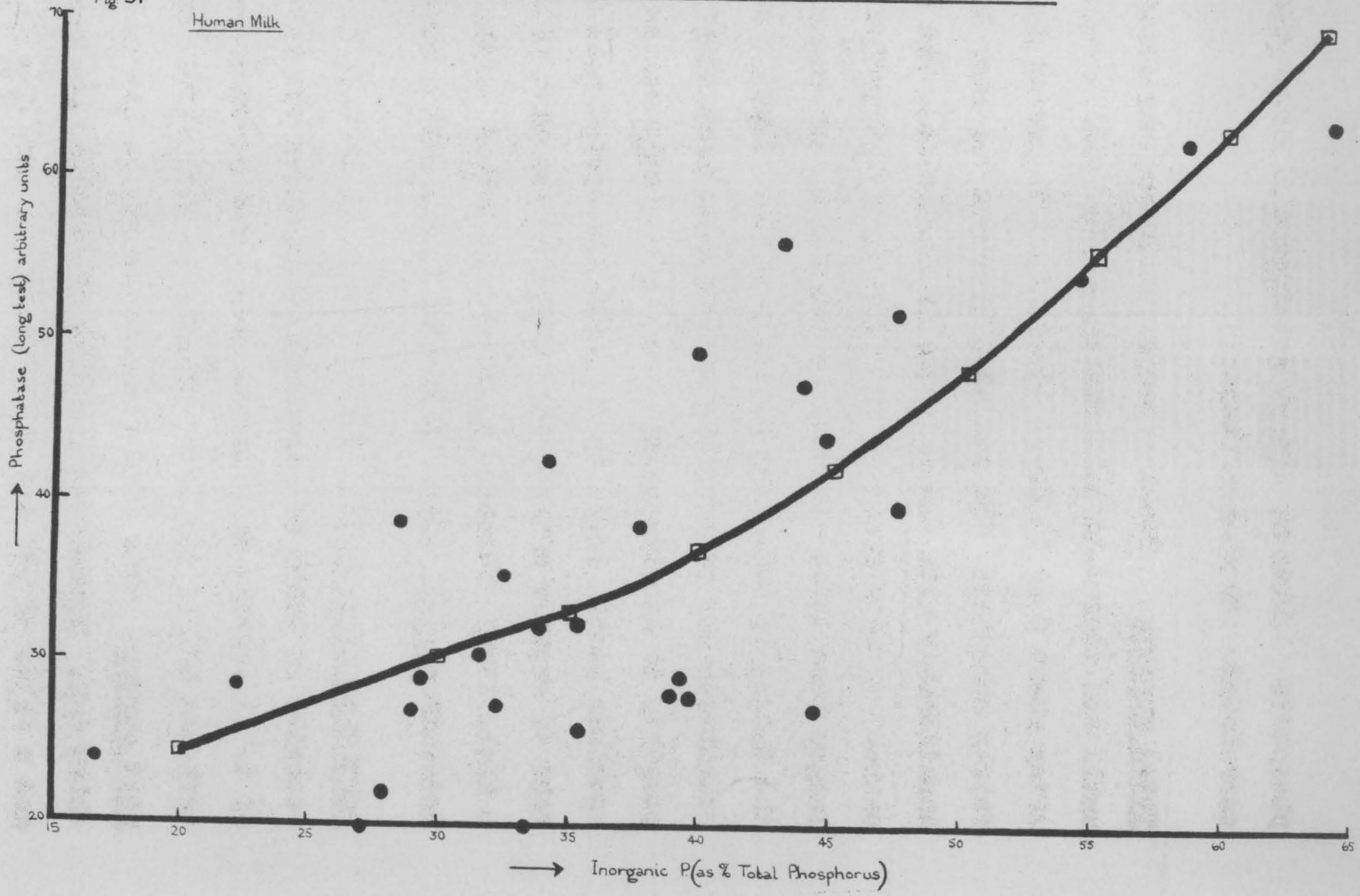

Correlation between Ester P and Phosphatase in Human Milk
Phosphatase (Jenz test) arbitrary units
70
60
50
40
30
20
12
0
1
2
3
4
5
6
7
8
9
10
Ester P (mg/100g milk)

Fig 31

Correlation between Inorganic P(as % Total Phosphorus) and Phosphatase.

Human Milk



phosphate (Tables 55 and 63 and Fig. 31) was positively correlated with phosphatase.

Total aneurin. Total aneurin (Table 58) was initially small and increased to three times its initial value after about 8 or 10 days. Thereafter the value remained fairly constant. The output of aneurin was also increased considerably with the progress of lactation. The aneurin content of milk found in the present investigation was larger than those reported by Kendall (1942) and from the U.S.A. by Roderuck, Williams & Macy (1945). The relatively low figures of the latter authors might possibly be attributable to the more highly refined American breed, since it is generally agreed that intake of aneurin effects the amount in the milk (Slater & Rial, 1942; Escudero & Pertusi de Esquef, 1944; Roderuck et al., 1945; Clements, 1949; Kon & Mawson, 1950)

Partition of aneurin. The partition of aneurin is also recorded in Table 58 along with values for the contents of total aneurin and phosphatase. Free aneurin contributed 28% of the total aneurin on the second day post partum. This figure was decreased to 15% on the third day. Thereafter the rate of decrease was smaller and a value of 12% was recorded on the 12th day post partum. There was a large increase by the 28th day, when the free aneurin was 41% of the total aneurin. The phosphorylated aneurin was proportionately decreased in the corresponding period.

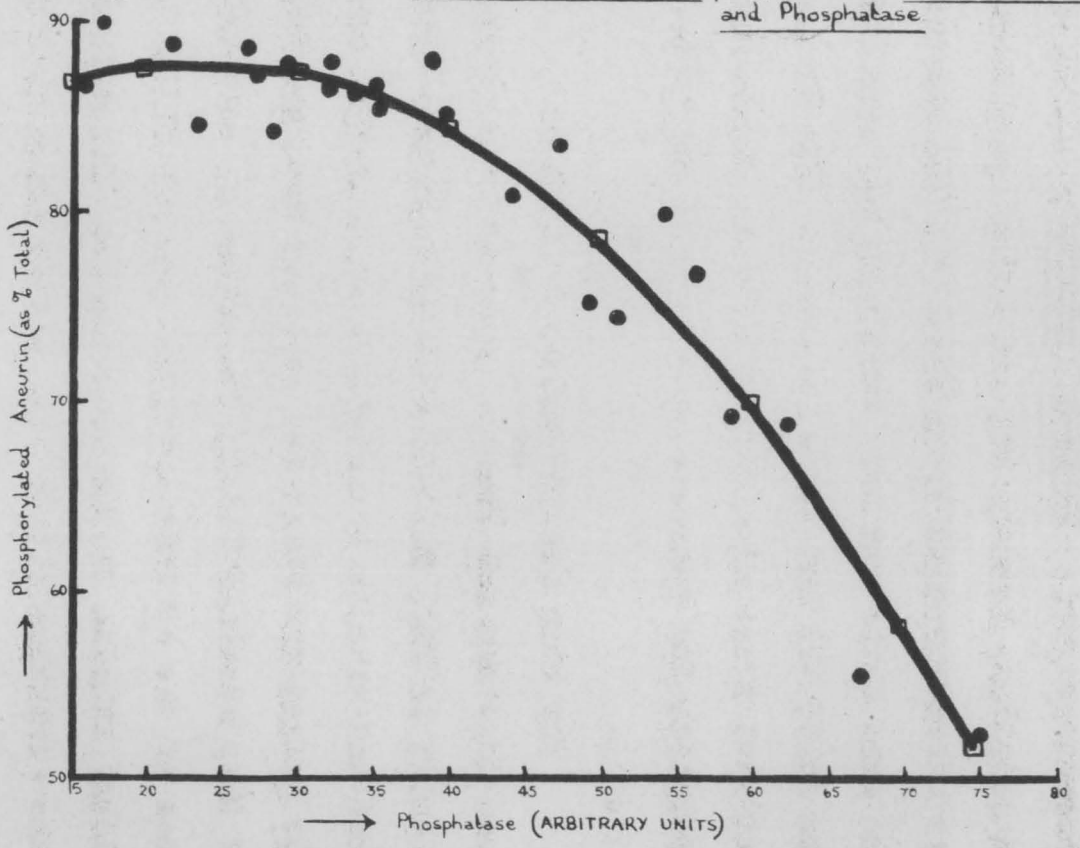
It was found that as much as 90% of the total aneurin could be present in human milk in the phosphorylated form. A value of 30% was found by Neuweiler (1941), but it should be observed that he did not record the days post partum on which his samples were collected. The present experiments showed that 52-69% of the total aneurin was present in the phosphorylated form even on the 28th day post partum.

Between phosphatase and free aneurin (expressed as a percentage of the total aneurin) there was a large positive correlation (Table 63). An equally large negative correlation was observed between phosphatase and phosphorylated aneurin expressed as a percentage of the total aneurin. The closeness of this correlation is shown graphically in Fig. 32 and an analysis of variance in Table 59. This inverse correlation between phosphatase and the ratio of phosphorylated to total aneurin has already been recorded for cow's milk (Chapter I). A positive correlation between phosphatase and free aneurin expressed as a percentage of the total aneurin was noted by Houston, Kon & Thompson (1940) for cow's milk, by Braude, Coates, Henry, Kon, Rowland, Thompson & Walker (1947) for sow's milk and by the present author in the milk of normal cows and cows treated with thyroxine or thiouracil (Chapter I).

Carotenoids and vitamin A. The results for carotenoids and vitamin A are recorded in Table 60. Chromatography showed that the fat from human milk contained xanthophylls,

Fig. 32

Correlation between Phosphorylated Aneurin (as % Total Aneurin) and Phosphatase



α- and β-carotene and lycopene, of which the last three were measured spectrophotometrically. That the red pigment present in human milk was in fact lycopene was demonstrated by comparing its properties with those of lycopene from tomato. The pigments from human milk and from the tomato showed only a single band when mixed and chromatographed. Their absorption spectra, in n-hexane solution, between 300 and 600 mμ were compared and are shown graphically in Fig. 29. The two curves are the same shape and have maxima at the same wave lengths (505, 475 and 448 mμ). These figures are concordant with those of Morton (1942) who found maxima of absorption for lycopene in petrol at 506, 474 and 444 mμ.

The mean concentration of carotene (α- and β-forms together) and vitamin A in the milk is shown graphically in Fig. 33. It will be seen that both the carotene and vitamin A content in milk dropped very rapidly during the first few days but from the 7th day onward they remained fairly constant. An analysis of variance of the vitamin A content per 100 ml. milk in individual samples on the 5th, 6th and 28th day, showed that the variation within days was so large that the differences between days from the fifth onward were not statistically significant. When the results were expressed as i.u./g. fat the drop in vitamin A continued significantly till the 8th day. Due to the high variation in the fat content of the milk between mothers

on the same day post partum, the total vitamin A in milk also varied within a wide range. The mean vitamin A on the third day post partum was found to be 418 i.u./100 ml. milk with a coefficient of variation ± 27%. The potency when expressed per g. fat was 162 i.u. on the same day and the coefficient of variation of this estimate was only ± 10%. Although total carotenoids decreased rapidly with the progress of lactation, the proportion of total carotenoids present as α- and β-carotene showed a small increase. Lycopene expressed as a percentage of the total carotenoids was decreased (Table 60). A most interesting aspect of Table 60 is the number of different forms of vitamin A which were found to be present in human milk. The amount present in alcoholic form varied from 18-54 i.u./100 ml. on the third day depending on the amount of fat present in the milk. The mean value of vitamin A alcohol per 100 ml. milk showed a gradual drop from the third to the seventh day post partum.

Both α- and β-carotene were found in human milk and were identified by their chromatographic and absorptive properties. Pure α-carotene and the mixtures of α- and β-carotenes obtained from carrots and from red-palm oil served to confirm the identity of the milk pigments. The results for the quantitative partition of carotene into α- and β-fractions are recorded in Table 61. The concentration of carotenes in the milk decreased as lactation advanced but the fall

in the α-fraction was greater than in the β-fraction. The ratio of β- to α-carotene consequently increased as lactation progressed.

In Table 62 the total vitamin A activity of milk both as a percentage of the milk and as a total output has been calculated. For this calculation it was assumed that 0.6 µg. of β-carotene was equivalent to 1 i.u. of vitamin A, that α-carotene had half the activity of β-carotene and that lycopene had no activity.

The author realises that these arithmetical transformations are open to serious criticism. Indeed it is obvious from the experiments of Cama & Goodwin (1948a) on the effect of desiccated thyroid on carotene metabolism in rats that the assignment of a definite factor for converting β-carotene to vitamin A is arbitrary, and the experiments described in Chapter III of the present thesis show that the same is true of cows and goats. One conclusion which arises from Table 62 is that the infant's natural intake of carotenes is small in comparison with its intake of vitamin A. As yet there is no evidence to indicate whether carotene has any function other than to act as provitamin A. Calculations based on the values in Table 60 show that each of these 28 infants could have obtained about 1000 i.u. vitamin A/day from their mother. This figure would be increased by only about 10% if all the biologically active carotenes ingested at the same time was converted to vitamin A. Most of the vitamin A was present in the form of vitamin A ester (Table 60).

Fig. 33

Carotenoids and Vitamin A in Human Milk

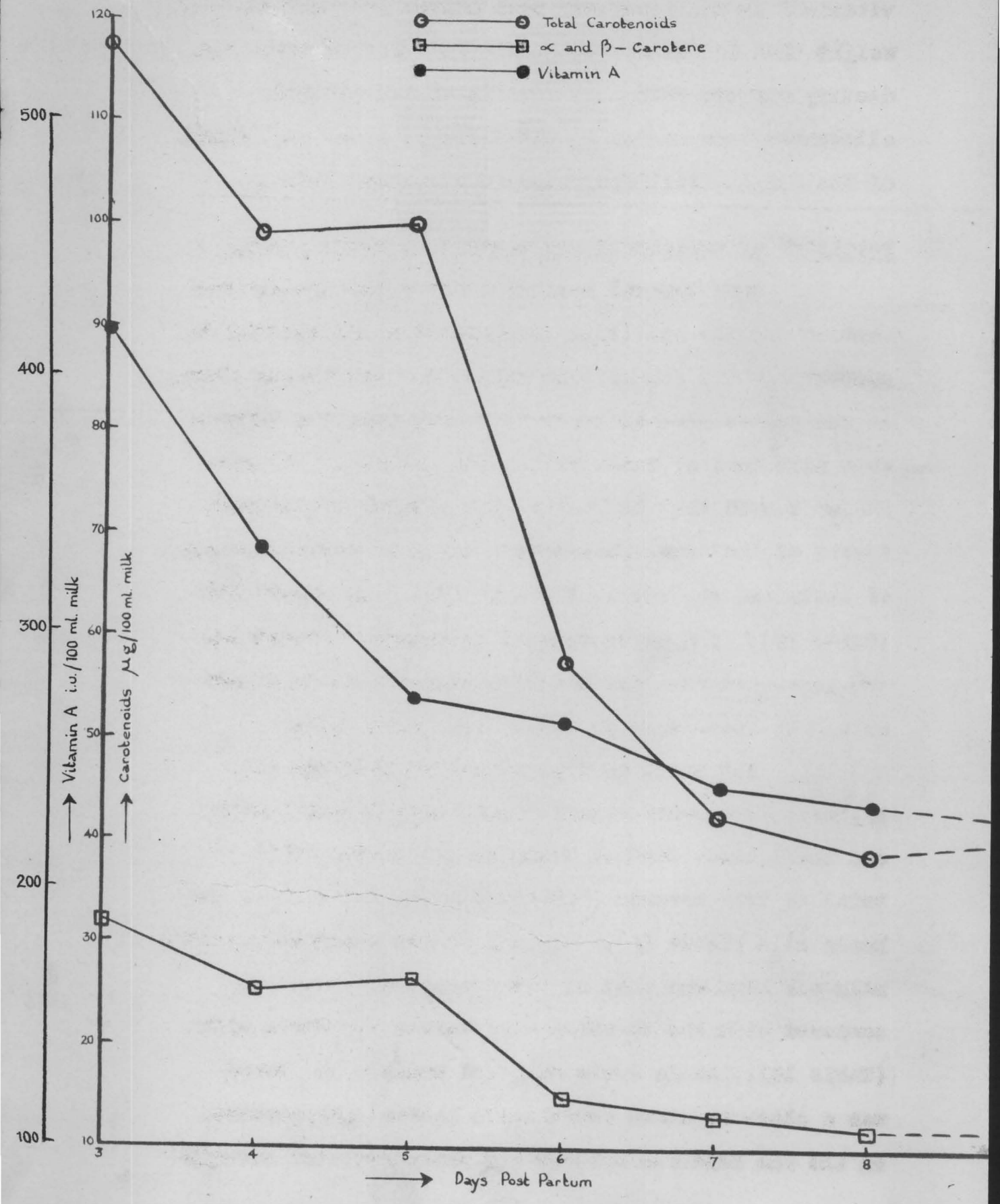


Table 62 shows that an infant's intake of vitamin A in all forms was much bigger per unit of body weight than is the average adult intake estimated from dietary surveys even on the basis of the liberal allowances recommended by the National Research Council of the U.S.A. (Jolliffe, Tisdale & Cannon, 1950).

Partition of phosphorus and aneurin in goat's milk.

Some typical results showing the phosphatase content and the partition of phosphorus and aneurin in goat's milk are recorded in Table 64. The enzyme titre of the goat's milk in early lactation compares favourably with that of human milk. The partition of phosphorus showed that in goat's milk ester-P contributed 24-35% of the total phosphorus, while at similar stages of lactation the cow's milk only contained about 14% (Table 18). The percentage of phosphorus present in the inorganic form was slightly higher than in human milk, but lower than in cow's milk (Table 18).

The total aneurin of goat's milk was 50% higher than (one-and-a-half times) that of cow's milk and three times that of human milk, but the ratio of total to free aneurin (Table 64) resembled that of the human milk (Table 58). Phosphorylated aneurin in goat's milk was high and that of the free aneurin was low compared with the corresponding values for cow's milk (Table 19). As in cow's milk and human milk, there was a close negative correlation between phosphatase on the one hand and ester-P and phosphorylated aneurin

on the other. The correlations of the enzyme with inorganic-P or with free aneurin were positive.

Table 54. The content of protein, fat and solids-not-fat (S.N.F.) of samples of human milk from 2 to 28 days post partum with the standard errors of the means

Days post partum	No. of samples	Protein (%)	Fat (%)	S.N.F. (%)
2	5	0.63 ± 0.06	3.4 ± 0.15	11.24 ± 0.73
3	11	2.01 ± 0.06	2.7 ± 0.28	9.77 ± 0.014
4	10	1.83 ± 0.05	3.0 ± 0.30	9.33 ± 0.16
6	12	1.74 ± 0.06	2.5 ± 0.10	9.79 ± 0.23
8	9	1.61 ± 0.05	2.9 ± 0.20	9.19 ± 0.22
10	1	1.82	3.1	9.39
12	1	1.67	2.8	9.52
28	3	1.74 ± 0.04	3.9 ± 0.27	9.62 ± 0.08

Table 55. The partition of phosphorus in human milk

Days post partum	No.of mothers	mg. P/100 g. milk ± standard error of the mean			Phosphatase (arbitrary units)
		Total	Inorganic *	Ester *	
2	4	13.3 ± 1.03	6.4 ± 0.26(48.1)	2.8 ± 0.52(21.1)	52.5 ± 3.7
4	10	15.9 ± 1.37	4.5 ± 0.45(28.3)	5.3 ± 0.54(33.3)	29.0 ± 9.8
6	10	21.7 ± 2.14	7.9 ± 0.59(36.4)	5.6 ± 0.90(25.8)	35.7 ± 4.2
8	8	18.6 ± 1.60	6.5 ± 0.62(34.9)	5.4 ± 0.60(29.0)	35.9 ± 3.9
10	1	15.7	5.3 (33.8)	6.0 (38.2)	32.2
12	1	20.4	8.0 (39.2)	6.8 (33.3)	29.5

* The figures in brackets show the percentage of the total phosphorus which was present as inorganic or ester phosphorus.

Table 56. Analysis of variance showing significant departure from linearity of the regression of ester P and phosphatase in human milk

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio
Total	28	4740.16	-	-
Linear regression	1	3651.68	3651.68	-
Deviation from linear regression	27	1088.48	40.31	-
Deviation from curvilinear regression	26	502.69	19.33	-
Curvilinearity	1	585.79	585.79	30.33***

Table 57. The amount of lipid P in human milk

Days post partum	Total P (mg./100 g.)	Lipid P (mg./100g.)	Lipid P as % of total P	Phosphatase (arbitrary units)
2	12.8 11.6	2.5 1.5	19.5 12.9	45.6 62.7
4	14.4 13.2 18.4 19.8	2.8 2.2 4.1 4.9	19.4 16.7 22.3 24.7	25.9 28.6 27.4 22.0
6	17.0 16.5 25.0 15.0	3.5 3.2 5.4 3.1	20.6 19.4 21.6 20.7	32.4 56.4 28.2 40.0
8	14.9 18.2 16.2 21.3	2.6 3.6 3.8 4.0	17.4 19.8 23.5 18.7	44.4 35.4 27.0 38.6
10	15.7	2.3	14.6	32.2
12	20.4	3.2	15.7	29.5

Table 58. The partition of aneurin and its output in human milk

Days post partum	Aneurin (μg./100 ml. fat free milk)		Ratio (Total/free)	Phosphatase (arbitrary units)	Milk yield (ml./day)	Output of aneurin in milk * (μg./day)
	Total	Free				
2	4.64	1.21	3.8	52.2	115	5
	5.91	1.49	4.0	49.5	185	11
	5.27	1.67	3.2	62.7	90	5
3	5.74	0.86	6.7	35.4	260	15
4	7.82	1.25	6.3	28.6	340	27
	6.95	0.79	8.8	22.0	700	49
	9.17	1.29	7.1	34.0	310	28
	7.52	1.26	6.0	47.6	260	20
	9.45	0.98	9.6	17.4	965	91
6	14.93	1.97	7.6	27.6	620	93
	10.73	1.68	6.4	23.6	465	50
	12.67	1.92	6.6	40.0	720	91
	11.28	1.41	8.0	32.4	440	50
	10.42	2.48	4.2	56.4	235	24
8	12.42	1.45	8.6	27.0	680	84
	9.94	1.34	7.4	35.4	530	53
	13.87	1.75	8.0	38.8	490	68
	14.56	2.98	4.9	54.5	305	44
	10.17	1.97	5.2	44.4	535	54
10	12.94	1.79	7.2	32.2	240	31
12	13.48	1.68	8.0	29.5	420	57
28	15.98	7.14	2.2	67.4	530	85
	13.77	4.28	3.2	58.9	540	74
	13.14	6.28	2.1	75.5	575	76

* In calculating aneurin output the small change in the volume of milk due to removal of fat was not taken into account.

Table 59. Analysis of variance showing significant departure from linearity of the regression of phosphorylated aneurin (expressed as percentage total aneurin) and phosphatase in human milk

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio
Total	23	2378.87	-	
Linear regression	1	1949.79	1949.79	
Deviation from linear regression	22	429.08	19.50	
Deviation from curvilinear regression	21	153.56	7.31	
Curvilinearity	1	275.52	275.52	37.68***

Table 60. Partition of carotenoids and vitamin A in human milk

Days post partum	Carotenoids *			Vitamin A			Fat	Yield of milk
	Total (μg./100ml.)	α- and β- carotene (as % of the total)	Lycopene (as % of the total)	Ester (i.u./100ml.)	Alcohol (i.u./100ml.)	Total (i.u./g.fat)	(%)	(ml./day)
3	121	24.4	50.1	236	25	145	1.8	130
	145	25.9	49.9	556	46	151	4.0	215
	137	28.9	48.3	333	42	187	2.0	117
	118	28.7	46.0	295	30	191	1.7	263
	110	38.7	31.2	484	54	138	3.9	363
	115	27.3	40.5	388	27	173	2.4	295
	99	22.3	52.4	282	18	177	1.7	150
	112	24.7	43.8	417	25	143	3.1	135
	126	24.8	40.0	507	36	151	3.6	205
	87	27.9	44.1	360	20	173	2.2	668
4	76	28.4	40.7	289	18	123	2.5	515
	112	25.9	44.3	417	35	94	4.8	187
	108	23.8	48.9	220	19	82	2.9	965
5	97	28.3	45.9	200	33	117	2.0	100
	88	22.3	47.3	287	22	82	3.8	651
	115	28.2	42.7	245	39	89	3.2	445
6	58	28.1	34.7	172	19	101	1.9	120
	67	25.4	34.8	295	24	80	4.0	235
	48	26.3	36.8	272	15	103	2.8	292
7	45	34.1	34.1	205	16	63	3.5	445
	39	28.6	40.6	245	14	78	3.3	500
8	41	32.1	17.9	181	16	66	3.0	535
	37	28.3	33.2	253	14	56	4.8	580
28	38	33.0	27.4	229	17	59	4.2	540
	34	29.0	39.7	207	12	52	4.2	530
	48	30.2	40.1	123	17	62	3.4	575

* Note: The total carotenoids include, besides α- and β-carotene and lycopene, other non-provitamin A carotenoids absorbing at 451 mμ, of which the chief is xanthophyll (lutein).

Table 61. Partition of carotene between α- and β-forms in human milk

Days post partum	Carotenes (μg./100 ml.)			Ratio β/α
	Total	β	α	
3	29.4	17.7	10.4	1.70
3	42.5	25.8	15.9	1.62
3	31.1	17.8	12.5	1.42
4	25.6	19.9	10.9	1.83
5	19.5	13.2	6.1	2.16
6	16.4	11.4	4.5	2.53
7	11.2	6.9	3.7	1.87
28	9.7	6.5	2.1	3.10

Table 62. Partition of vitamin A activity in human milk between carotenes and vitamin A, milk yield, fat percentage and output of total vitamin A activity in all forms

Days post partum	Vitamin A (i.u./ 100ml.)	Vitamin A activity due to α-carotene (i.u./ 100ml.)	Vitamin A activity due to β-carotene (i.u./ 100ml.)	Total vitamin A potency (i.u./ 100ml.)	Proportion of vitamin A activity due to carotene (%)	Milk yield (ml./ day)	Fat (%)	Total output of vitamin A (i.u./ day)
	(a)	(b)	(c)	(a+b+c)				
3	261.2	9.1	29.6	299.9	12.9	130	1.8	390
3	537.1	13.3	43.1	593.5	9.5	363	3.9	2154
3	543.3	10.4	29.7	583.4	6.9	205	3.6	1196
4	238.7	9.1	33.2	281.0	15.1	965	2.9	2712
5	309.8	5.1	22.0	336.9	8.0	651	3.8	2193
6	191.2	3.8	19.0	214.0	10.7	120	1.9	257
7	258.4	3.1	11.5	273.0	5.3	500	3.3	1365
28	218.8	1.8	10.9	231.5	5.5	530	4.2	1227

Table 63. Correlation between phosphatase and various other constituents of the milk

Variate	Correlation coefficient	No. of paired observations	P
Ester phosphorus	- 0.8791	29	<0.001
Ester phosphorus as % total P	- 0.8542	29	<0.001
Inorganic phosphorus as % total P	+ 0.8078	29	<0.001
Lipid P	- 0.5526	16	<0.05 >0.02
Lipid P as % total P	- 0.5171	16	<0.05 >0.02
Free aneurin	+ 0.7306	24	<0.001
Free aneurin as % total aneurin	+ 0.9056	24	<0.001
Phosphorylated aneurin as % total aneurin	- 0.9053	24	<0.001

Table 64. The partition of phosphorus and aneurin in goat's milk at early lactation

Goats	Total P (mg./100 g.milk)	Percentage of total P present as		Aneurin (μg./100ml. fat free milk		Ratio of total aneurin to free aneurin	Phosphatase (arbitrary units)
		Inorganic P	Ester P	Total	Free		
Anna	76.3	46.3	29.8	55.8	6.6	8.5	35
Betty	99.3	48.4	30.2	40.6	4.7	8.6	34
Bluebell	101.5	46.6	29.3	65.2	7.2	9.1	32
Diana	84.9	49.4	27.4	67.8	8.9	7.6	41
Heather	79.3	52.3	23.6	78.9	12.1	6.5	48
Judith	83.3	47.3	30.0	72.1	8.5	8.5	37
Mazy	109.2	48.3	29.4	68.4	7.8	8.8	35
Miranda	96.0	47.5	29.2	66.2	8.1	8.2	37
Susan	113.9	43.5	35.0	96.2	9.3	10.3	21

Discussion

In these observations human lactation was found to differ from that of the cow in respect of secretion of protein. Thus the human colostrum studied had a smaller concentration of protein than later milk, while cows' colostrum was richer in protein than was later milk. The concentration of protein increased as lactation proceeded whereas in the cow the initially protein-rich colostrum gave way to milk of poorer content. These differences between human and cow's milk may perhaps be correlated with the richness of cow's colostrum in globulin with its accompanying content of antibodies. In the human such antibodies are chiefly acquired *in utero* thus lowering the neonatal globulin requirements. Moreover the calf grows more rapidly than the human infant and therefore needs a liberal neonatal intake of calcium phosphate for bone growth. This in turn necessitates a larger content of casein in the milk in order to prevent the precipitation of calcium phosphate.

In cow's milk typical figures for the concentration of nitrogen and phosphorus are 0.6% and 0.1%. Thus N/P = 6:1. The present figures give N:P for human milk as being 0.300:0.015 or 20:1. Observations reported in Chapter I of this study showed that significant decreases in the ratio occurred when cows were treated with thyroxine, while the ratio was increased by treating cows with thiouracil (p.29). It therefore

appears that the ratio of N:P in milk is a characteristic of the species but that it is also dependent on the thyroid status within the species.

In the present experiments phosphatase in human colostrum and milk was found to be low but varied in a manner similar to that in other species. By analogy therefore between the bovine and the human species it would be expected that the much smaller phosphatase titre of human milk would be associated with a much greater proportion of ester-phosphorus since the enzyme has been shown to be inversely correlated with it. This is indeed the case, for in the present investigations human milk on the twelfth day post partum had more than 30% of the phosphorus as ester (Table 55), whereas in cow's milk this figure was about 15% at a similar stage of lactation (Table 18). A comparison of the results of the partition of phosphorus of goat's milk (Table 64), cow's milk (Table 18) and human milk (Table 55) shows that in respect of phosphatase and ester-P goat's milk resembles human milk and not cow's milk in spite of the fact that the concentration of total P in goat's milk is much the same as in cow's milk. The enzyme titre of the goat's milk in early lactation also compares favourably with that of human milk. These observations provide some scientific background to the popular belief that goat's milk is superior to cow's milk for infant feeding.

Some correlations recorded in Table 63 show that in human milk lipid-P and phosphorylated aneurin are negatively correlated with phosphatase, while in-

organic phosphate is positively correlated with phosphatase. These correlations are consistent with the hypothesis that ester-P is the intermediary in metabolism between inorganic phosphate and lipid and casein phosphorus. The intermediary ester-P may perhaps be synthesised in the mammary epithelium by phosphatase from inorganic phosphate.

The large amount of vitamin A found to exist in the alcoholic form in this investigation (12-54 i.u./100 ml. milk) indicates some difference between the human and the cow which merits further investigation. Ganguly, Kon & Thompson (1947) and Parrish, Wise & Hughew (1947) found only 3-10 i.u./100 ml. cow's milk or colostrum. In the investigations reported in Chapter III, 4-6 i.u. were found in cow's milk. The great variation in total vitamin A from sample to sample was perhaps partly due to failure on the part of some of the mothers to take the vitamin capsules supplied by welfare centres and hospitals. Its chief cause, however, was undoubtedly the great variation in the percentage of fat in the milk (Table 60). There was a considerable reduction in the coefficient of variation when the results of vitamin A were expressed as i.u. vitamin A/g. fat.

The large amount of carotenoids found to be present in human colostrum and transitional milk is of doubtful biological significance. Chromatographic study showed that most of the carotenoids were the biologically inactive xanthophylls and lycopene (Table 60). The

occurrence of lycopene in human milk (Thompson, Kon & Mawson, 1942) and in cow's milk has previously been observed and it is known to be of dietary origin (Thompson, Ganguly, Mawson & Kon, 1949). The extent to which tomatoes and swede turnips contributed to the occurrence of lycopene in the present samples is not known. The occurrence of β-carotene is attributable to any greenstuff but its occurrence with α-carotene is probably due to the presence of carrots in the diet.

Summary

Samples of normal milk from mothers delivered in the Aberdeen Maternity Hospital were analysed with the following results:

1. The percentage of protein was very small (0.63) on the second day post partum, but rose to 2.01 the next day and remained at about that level thereafter.

2. Percentages of fat ranged from 2.5 to 3.9 and were much more variable than those of protein.

3. Solids-not-fat ranged from 9 to 10% with an initial figure of about 11.2%.

4. Inorganic, ester and lipid P were closely correlated with the phosphatase content of the milk. Inorganic P was positively, and ester-P and lipid-P were negatively correlated with phosphatase. The phosphatase contents of the milk were initially large, and decreased as lactation progressed.

5. The N/P ratio in the human milk was 20 compared with only 6 for cow's milk.

6. Total aneurin increased steadily with the progress of lactation. Phosphorylated aneurin behaved like ester-P in respect of its relationship to phosphatase with which it was closely but negatively correlated.

7. On the basis of the present analyses an infant's total intake of vitamin A (measured in i.u.) could vary from 390 to 2112. The larger figure represents an enormous intake judged by adult standards if infant and adult are compared on a weight for weight basis.

8. The vitamin A activity of human milk was due to vitamin A itself, though the contribution from biologically active carotenes varied from 5 to 15% of the total. Of the vitamin A more than 90% was in the form of ester.

9. Lycopene, which is not a precursor of vitamin A, formed a quarter to a half of the total carotenoids. The similarly inactive xanthophylls were also present but no attempt was made to estimate them quantitatively.

10. The results for human milk are compared with those of cow's milk reported in earlier chapters.

11. Some typical results of the phosphorus and aneurin distribution in goat's milk have been recorded and their similarities with human milk discussed.

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